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## SYSTEMATIC SEROLOGY: A CRITICAL APPRECIATION[1]

(Twelve figures)

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## PART I. SYSTEMATIC SEROLOGY AND ITS RELATION TO GENERAL BIOLOGY

### I. OBJECT OF THIS REVIEW

THE purpose of this survey is to present a critical analysis of the theory and fact of systematic serology as applied to animals with their meaning in relation to general biology. There is no survey of this kind available, for such an analysis must contain more than a tabulation of the results of testing antisera with a variety of antigens. There must be, in addition, a statement of the importance and limitations of systematic zoölogy, for the relative importance of any serological analysis of animal relationship depends, first, on the importance of knowledge of animal relationship itself and, second, on the need for some additional basis for its determination. Above all, there is need for a clear description and thorough criticism of the ways in which serological comparisons are made, for the literature is full of errors resulting from comparisons made by noncomparable methods upon nonequivalent and even noncorresponding materials.

### II. THE TASK OF SYSTEMATIC SEROLOGY AND THE MEANING OF TERMS

The task of systematic serology is the investigation of the possible uses of serological reactions in the determination of animal relationships and the application of whatever techniques may be found useful for this purpose as effectively and extensively as can be done. To discuss this task effectively and to evaluate the data obtained by means of serological reactions requires the comprehension of certain terms—let us then "come to terms."

[1] *Editorial note.*—This is another in the growing series of invited synthesizing papers in *Physiological Zoölogy.*

[2] Publication of the Bureau of Biological Research, with the assistance of General Scientific Project, Division of Professional and Service Projects, Work Projects Administration of New Jersey. A portion of the expense of publication has been borne by Rutgers University.

Agglutinins—Antibodies capable of causing a clumping of formed antigens such as R.B.C. or bacteria.

Agglutinin reaction—The reaction between agglutinins and the formed antigens which react with them.

Animal relationship—The systematic position of animals as determined by all the available knowledge of their fossil history, geographic distribution, genetic nature, comparative morphology, and physiology.

Antibody—A substance usually produced in response to the injection of proteins capable of combining with and reacting upon the homologous antigens and other chemically related substances.

Antigen—A substance capable of inducing the formation of antibodies under appropriate conditions.

Antiserum—A serum containing antibodies.

Flocculation technique—That manner of performing the precipitin reaction which involves mixing the antigens and antisera.

Genetic relationship—Organisms which have had common ancestry possess genetic relationship.

Hapten—A substance, not antigenic, but capable of combining with antibody as antigen does.

Heterologous reaction—The reaction between an antibody and any antigen (or hapten) other than the antigen used in its formation (we propose to call these "secondary reactions").

Homologous reaction—The reaction between an antiserum and the particular kinds of antigens used in its formation (we propose to call these "primary reactions").

Precipitins—Antibodies capable of combining with and reacting upon antigens or haptens used in solution.

Precipitin reaction—The reaction between precipitins and the antigens or haptens capable of reacting with them.

Ring test—That manner of performing the precipitin reaction which involves carefully layering the antiserum under the antigen dilutions (synonyms are "Schichtprobe," "contact method," "interfacial technique").

Reciprocal reactions—A set of reactions, one pair concerning anti-*A* tested with *A* and *B*, and the other pair anti-*B* tested with *B* and *A*.

Serology—That division of biology which deals with the nature and reactions of antigens and antibodies.

Serological correspondence—As applied to antigens it includes all antigens capable of reacting with an antiserum to any one of them. As applied to antibodies or antisera it includes all antibodies or antisera capable of reacting with a given antigen or hapten.

Serological equivalence—Whenever the serological correspondence is exact, we may speak of serological equivalence.

Specificity—That property of serological reactions in which the primary (homologous) reactions are more intense than the secondary (heterologous) reactions.

Titer—The end point of a serological reaction, i.e., the highest dilution of antigen or antibody capable of reacting with a fixed amount of antibody or antigen, respectively.

## III. TECHNIQUES USED IN SYSTEMATIC SEROLOGY

The serological reaction most used in systematic serology is the precipitin reaction. There are two principal methods of performing the precipitin reaction, i.e., the flocculation test and the ring test.[3] In the flocculation procedure the antigens and antibodies

[3] The old term "ring test" is objected to by some as a misnomer. However, under certain conditions, as noted first by Ascoli (1902), the zone of contact between antigen and antiserum does have the appearance of a ring. The terms "contact method" or "interfacial technique" are certainly no better and no more distinctive, for what colloidal reactions do not involve contact and interfaces? The term "Schichtprobe" or layering test is the most accurate but is not commonly used in the literature. Under the circumstances we feel justified in using the original term "ring test."

are mixed in certain proportions, and the precipitation which results may be described in qualitative terms or measured as weight, nitrogen content, volume of precipitate, or turbidity of the reagent mixture. The reagents—antigen or antiserum—may be used in one proportion only, or, more commonly, a constant amount of antiserum may be mixed with increasing dilutions of antigen, and the titer or end point thus determined.

## PROCEDURE

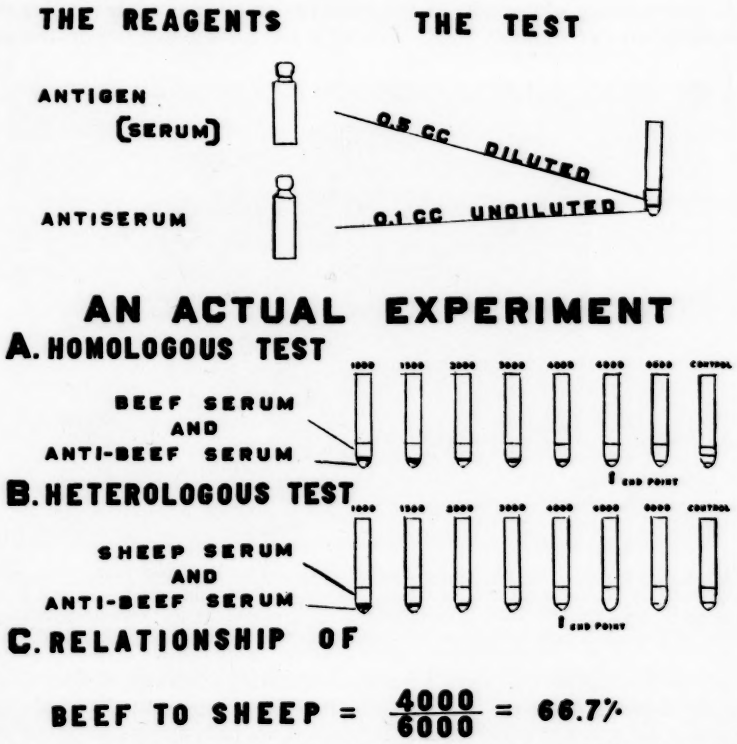


FIG. 1.—A diagram to illustrate the procedure in the ring test. One tenth-ml. of antiserum is carefully layered under ½ ml. of increasing dilution of antigen in buffered saline. A white layer of precipitate is evidence of a positive reaction. The titer is the highest dilution of antigen capable of reacting within the time limit, and the diagram shows the relative sensitivity of an antibeef serum to beef and sheep sera (from Boyden, 1934, p. 521).

In the ring-test procedure the antiserum is carefully layered under the antigen dilution, and, most commonly, constant antiserum is reacted with increasing antigen dilutions until the end point or titer is determined. Here again the reaction may be performed with only qualitative results, or it may be performed in a quantitative manner involving known amounts of antigen. For a diagram illustrating the ring test see Figure 1.

Now it should be pointed out at once that what is measured even in the most accurate quantitative determinations of the precipitin reaction is different in the ring test from what is measured in some types of flocculation test. Thus what is measured in quantitative ring tests is the *sensitivity* of the antiserum, i.e., how little of a given antigen is needed to give a reaction with the antiserum titrated: This titer can also be determined by flocculation test, where the end point may be (*a*) the highest dilution of antigen showing a flocculent precipitate or (*b*) the highest dilution of antigen showing a greater turbidity than the antiserum control. These titers indicate only *sensitivity;* they do not measure the *amounts* of reaction concerned. To make this distinction clear let us consider the measurement of intensity of the precipitin reaction as made possible with the "photronreflectometer" (Libby, 1938). Figures 2 and 3 show this new instrument and

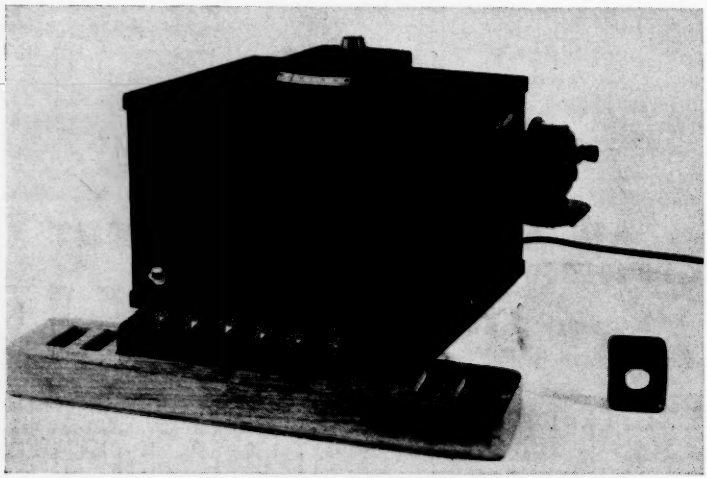

FIG. 2.—A photograph of the photronflectometer developed by Raymond Libby. The instrument operates on ordinary house current and provides a constant beam of light, which is passed through the cell containing the reagents. Turbidity in the cell causes diffraction of the light onto sensitive areas of the photoelectric cell, which in turn deflects the galvanometer needle (unpublished photograph).

explain its mode of operation. Thus, when a precipitating antiserum is reacted with increasing dilutions of an antigen and the turbidities developing over the whole range of proportions of antigen and antiserum are summated, we have a measure of the *amounts* of reaction taking place and not merely an indication of the sensitivity of the antiserum. We submit that the summation of turbidities, photoelectrically measured, is a more adequate estimate of the precipitating capacity of an antiserum combined with a given antigen than any sensitivity method. What has been said of the photronreflectometric measurements would apply also to the determination of the volumes of precipitate or the nitrogen contents of washed precipitates. One further point must be clearly understood. The combination of precipitin with a given antigen shows the phenomenon of optimal proportions. This means that the amount of precipitate obtained with constant amounts of antiserum and increasing amounts of antigen rises from zero with a subminimal

amount of antigen to a maximum value where antigen and antibody occur in nearly equivalent amounts yielding a maximum amount of precipitate, and then the amount of precipitate falls off again to zero in the zone of considerable antigen excess. The occurrence of this phenomenon of optimal proportions makes it necessary that either (1) whole curves be compared or (2) corresponding points on such curves be compared.

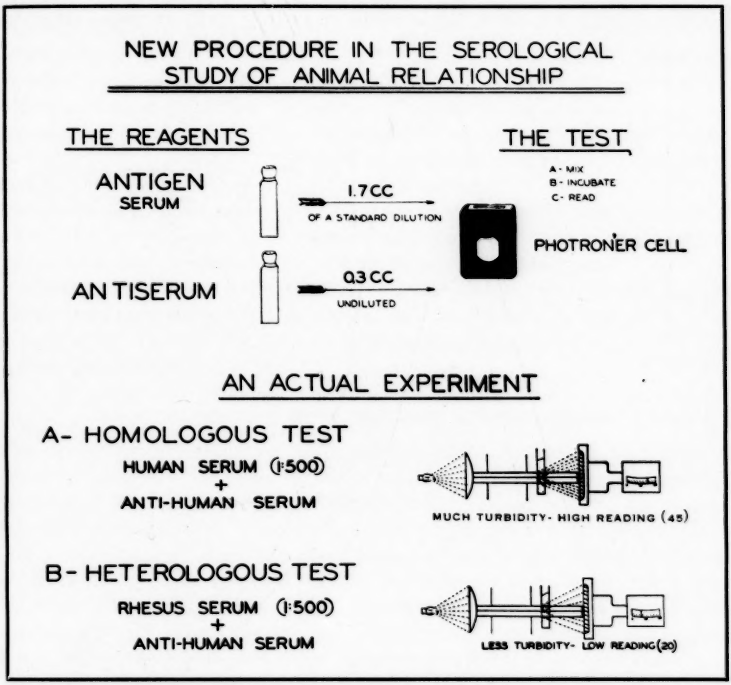


FIG. 3.—A diagram to show the use of the photronreflectometer in serological comparisons. The galvanometer readings are proportional to the turbidity which develops in the cell containing the reagents. Using constant antiserum and increasing dilutions of antigen, the entire precipitating capacity of each antiserum is determined over the whole range of antigen dilutions (unpublished diagram).

Now, since the determination of these *corresponding* points can be made only after the whole curve is obtained, it is obvious that actually the proper basis for comparison of the reactions of a given precipitating antiserum with a variety of antigens is the whole curve representing the total reactivity of each antiserum with each antigen. Any lesser basis of comparison such as the measurement of the amounts of precipitate obtained from the interaction of some single proportion of antigen and antibody could lead to serious error, owing to the fact that the single proportions were not taken from corresponding parts of their own curves.

An understanding of this important technical consideration may be gained from an examination of Figure 4, which shows the results of measuring the turbidities obtained

with constant amounts of antiserum and increasing dilutions of antigen. The curves all show optimal proportions, but the optimum points for the different curves come at different proportions of antigen and antiserum! Thus no single selected antigen concentration can represent corresponding points on these curves, obtained under standard conditions with equivalent amounts of antigen. Furthermore, when the antigen concentrations were unknown, as in most of the early serological work, and only one proportion of antigen and antibody was used, the chances of getting really corresponding data were

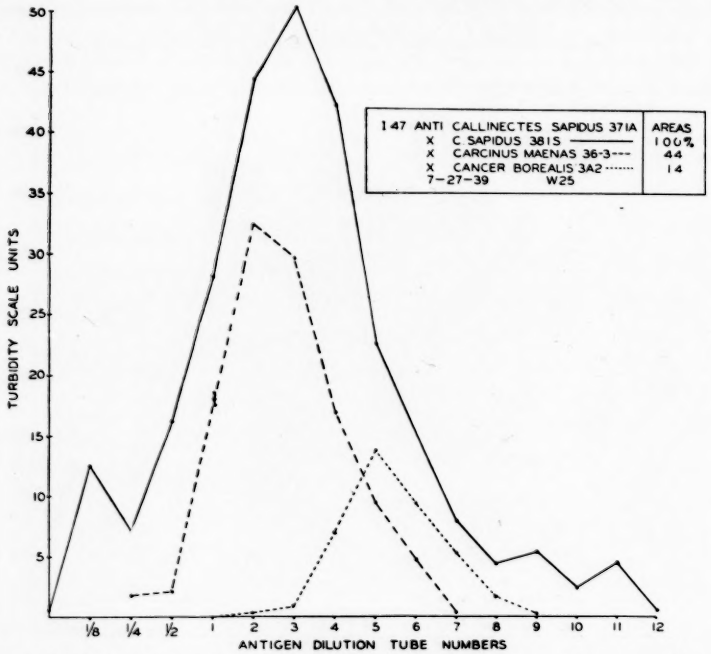


FIG. 4.—The results of titrating with the photronreflectometer an antiserum against *Callinectes sapidus*, tested with the sera of *C. sapidus*, *Carcinus maenas*, and *Cancer borealis*. The galvanometer readings are indicated along the ordinate, the antigen dilutions along the abscissa. Tube 1 contains 1 part of protein in 500 parts of saline, and each succeeding tube contains half as much antigen as its predecessor (from Boyden, 1939).

practically nil. These considerations must be borne in mind if we are properly to evaluate the serological data in the literature, for these matters apply to all attempts to measure the amounts of precipitation obtained, whether the technique used involves volumes, nitrogen contents, weights of precipitate, or turbidity measurements.

The agglutinin reaction comes next to the precipitin reaction in the extent of its application to problems of systematic serology in animals. Fundamentally, it is of the same nature as the flocculation precipitin technique, where the titer or sensitivity of the antiserum is determined. The antigen is in the form of suspensions of cells or cell fragments, however, and the antiserum is tested in a series of dilutions with constant antigen.

## IV. WHAT CONSTITUTES GOOD RESEARCH IN SYSTEMATIC SEROLOGY?

With this brief discussion of techniques before us we are in a position to outline tentatively the minimum requirements needed in carrying on and reporting research in systematic serology. These requirements are as follows: (*a*) The antigens used must be of known origin, relatively simple, and prepared in similar ways. (*b*) The antisera used must be prepared in such a way as to have similar and useful properties. (*c*) The technique used must be at least semiquantitative in nature and clearly described. (*d*) Sufficient repetitions of tests of the same kind must be made to establish the ordinary range of error which applies to the data presented.

The data obtained must then be judged on the following grounds:

*a*) *Consistency.*—(1) Are the data significant in relation to the range of error applicable to them? (2) Are antigens, shown to be very similar or indistinguishable by a suitable specific antiserum, treated similarly by all antisera?

*b*) *Comparability of reagents.*—(1) Were the antisera made and tested in the same ways? (2) Were the antigens obtained in the same way and used in equivalent amounts? (3) Did the homologous reactions exceed all heterologous reactions?

*c*) *Systematic significance of the results.*—(1) Do the reactions parallel the genetic relationship of animals where that is known, i.e., in the case of species and hybrids of known origin? (2) Do the reactions parallel systematic relationships where the latter seem well substantiated?

## V. FUNDAMENTAL PRINCIPLES OF SYSTEMATIC SEROLOGY

If the above procedures may be considered good serological technique from the standpoint of modern systematic serology, what, we may ask, is such serological technique good *for?* We come now to the theory underlying serological reactions as guides to systematic relationships. The fundamental principles are as follows: (*a*) The biochemical composition of organisms must be considered in any complete natural system of classification. (*b*) Some complex organic constituents of protoplasm are conservative hereditary traits. (*c*) Chemical similarities in antigens and haptens are revealed by adequate serological technique, and the chemical similarity is proportional to the intensity of the serological reactions. Are these principles valid? Let us examine the considerations and the evidences which support them.

*a*) There are probably very few zoölogists who would openly deny the validity of principle (*a*), though some "dyed-in-the-wool" morphologists might belittle their importance. It is true that, after more than a century of intensive comparative morphology and phylogeny, we still have whole phyla of uncertain systematic position, and no amount of similar morphological investigation is likely to determine their proper systematic position. The fact is that there has been too much evolution before us, that these phyla (if they ever did have common ancestry with other organisms, which cannot be proved) have become too diverse to allow us to classify them certainly in a scheme with genetic implications attached to their systematic positions. We need an independent, more objective, and more quantitative basis for the determination of these, of all, animal relationships. What can be more objective than chemical description? From the standpoint of objectivity alone, biochemical evidence regarding the natures of organisms may ultimately outweigh all other bases of classification, provided only that such biochemical comparisons deal with conservative inherited traits.

*b*) What is the evidence regarding the hereditary nature of the capacity to develop antigens during development? In the first place the physical continuity of succeeding generations guarantees that each generation develops with a definite kind of protoplasm, which actually is a relatively large or small fragment of the protoplasm of a preceding generation. The great principle of biogenesis, therefore, and its mechanism of reproduction provide for the inheritance of the original biochemical constitution of each organism. But, besides this, new antigens as well as new structures appear at definite developmental stages, and they are both determined by heredity in essentially the same ways. Thus in the chick embryo, *chick* serum proteins—not duck, not goose, proteins, though similar to them—appear at an early stage. Lens proteins appear, and the capacity to develop any lens and any lens proteins is inherited, as modern knowledge of the inheritance of certain eye defects indicates. In the higher apes and man the blood groups are inherited in a simple way, so that here the capacities to develop the antigens and the antibodies are both determined by the genetic constitution of the organism. In comparing horse and ass and mule, suitable antisera of a sufficiently discriminating nature distinguish horse and ass and place the hybrid mule in between its parents (see Figs. 10, 11). Serological qualities are therefore strongly inherited, and, as a matter of fact, they appear to be remarkably immune from environmental influences.

*c*) Finally, what is the evidence that serological reactions parallel the chemical similarities of antigens? The evidence is overwhelming. It is ably and fully presented in Wells (1929), in Landsteiner (1936), and in Marrack (1938), and there is certainly no need of detailing it again. These are, very briefly, the essential facts as they bear on the determination of animal relationships.

1. The injection into an animal to be used as antibody producer (commonly the rabbit) of a single antigenic substance, such as purified haemocyanin, haemoglobin, or crystalline egg albumin, results, under proper conditions, in the development of an antiserum containing one or more kinds of antibodies, which react more strongly with the homologous antigen than with other substances in comparable amounts.

2. Antisera produced in the common manner (5–10 mg. protein per kilogram body weight of the rabbit in first dose followed by two or three successively doubling doses on alternate days) are characterized by their quantitative specificity, i.e., they react to nearly the same degree with chemically similar antigens—such as the crystalline egg albumins of hen and duck, the haemocyanins of closely related species of Crustacea such as *Cancer borealis* and *C. irroratus*, the serum proteins of horse, ass, and mule. The same antisera react much less strongly with the less similar corresponding antigens of more distantly related species.

3. Where the chemical constitution of antigens and haptens is well known, it has been conclusively shown that serological specificity depends upon chemical constitution. A slight alteration in the chemical constitution of antigens gives a slight change in specificity; a drastic change in chemical constitution of the antigens may completely alter the serological reactions of these antigens.

We may conclude, then, that the fundamental principles of systematic serology are valid and that they should be useful in aiding us to reach consistent interpretations of animal relationships and definitive natural classifications. But is there any real need of systematic serology? Cannot the problems of animal relationship be solved by the older methods?

## VI. THE NEED OF INVESTIGATIONS IN SYSTEMATIC SEROLOGY

The need for systematic serology can be demonstrated in two ways: (*a*) by calling to mind the fact that we still have whole phyla of "uncertain systematic position," notwithstanding more than a century of intensive morphological investigation, and (*b*) by an examination of the generally accepted principles of systematic zoölogy. The first point speaks for itself; let us proceed to the second.

Here is a set of principles based on the works of Darwin, Haeckel, Gegenbaur, Montgomery, Ferris, and others, which were submitted to a group of American systematic zoölogists and received their general approval.

1. All animal relationship is genetic, i.e., a result of the phenomena of protoplasmic activity summarized under the principles of biogenesis (life from life), heredity, and variation.
2. Animal relationship is and should be determined chiefly by morphological comparison (of fossil, developmental, or adult structures); and the greater the degree of morphological similarity, the greater the degree of genetic relationship.
3. The central concept of comparative morphology is homology, i.e., a fundamental similarity in the position and form of corresponding organs or parts of the body of different animals, which is due to common heritage. "Blood relationship becomes dubious exactly in proportion as the proof of homologies is uncertain."
4. The proper basis of comparison in the analysis of racial descent is the whole individual during its whole life-history. Within this unit, selected stages must always be compared only with corresponding ontogenetic stages.
5. Those characters are of greatest value as guides to kinship which are the most conservative.
6. The goal of taxonomy is the expression of phylogenetic relationships.

Now are these principles valid? Are they adequate? By following them can a systematic zoölogist reach a truly natural and definitive classification, i.e., one which groups organisms according to their essential natures?

Take the first principle, "All animal relationship is genetic." The most common implication of this statement would be that all systematic relationship is genetic or that all animals are genetically related. Neither can be proved. Actually, we shall never be able to prove the common ancestry of all animals, and therefore we have no right to state as a fundamental principle what must always remain an assumption. It is true that we have adequate reasons for assuming common ancestry *within* many systematic categories; but to state that all animals are genetically related goes far beyond verifiable truth. Confidence in the findings of systematic analysis cannot be developed, if the habit of making such exaggerated claims prevails.

The second principle contains truth. That animal relationship is *now* determined mainly by morphological comparison (of fossil, developmental, or adult structure) is certainly true, but to add that it *should be* so determined forever is again to go beyond the facts. It may also be generally true that the greater the degree of morphological similarity, the greater the degree of genetic relationship, but there is no way of determining "genetic relationship" precisely, for there are all kinds and degrees of it and in the absence of pedigree records *any* degree of genetic relationship at all is implied from the natures of the organisms compared. Furthermore, precise estimation of morphological similarity is difficult and seldom accomplished, and in complex organisms there is always the difficult problem of weighting different sets of characters. We should base our analysis of the nature of organisms not only on their gross and microscopic structural

expressions but on their biochemical constitution, and it may even be true that eventually biochemical analysis will have an equal or greater weight in systematic zoölogy than will morphology.

The third principle is sound to the extent that morphological homology is a genetic reality in spite of the claims of some geneticists to the contrary (see discussion by Boyden, 1935). The real difficulty, however, lies in the attempt to determine the "end point" of the relationship of homology. We have no certain means of doing this at present, and consequently the views regarding the more inclusive homologies of certain structural elements (germ layers, gut, coelome, etc.) are conflicting. The worst aspect of current discussions on homology is their confusion in the relations between homology and analogy, for commonly homology is used as though it were opposite in meaning to analogy, whereas actually homology has always signified an essential structural resemblance and analogy—since Owen (1843), a functional resemblance—and these terms are not opposites in any proper usage of the terms. The natural opposite of homology is nonhomology and of analogy is nonanalogy, and the same organs are usually both homologous and analogous, as Owen clearly indicated nearly one hundred years ago. Principles 4 and 5 relating to comparing whole individuals and whole life-cycles and the weighting of characters by relative conservatism are essentially correct, yet their application to systematic zoölogy is far from simple or complete. A large amount of systematic work has been done without including entire individuals, much less entire life-cycles. As far as conservatism of characters is concerned, it is difficult to assign relative weights to morphological characters on any basis; and the determination of the degree of conservatism of morphological characters is difficult even where fossils are abundant.

Finally, it is stated, and seems to be generally agreed, that the goal of taxonomy is the "expression of phylogenetic relationships." However, in the absence of pedigrees phylogenetic relationship is inferred from the morphological expression of existing and preexisting types. Actually, both phylogeny and classification are based on the revealed characters of the organism, and of the two the classification is the more necessary and biologically significant. We are glad to have available all the reasonably correct phylogenies that can be proposed, but we *must* have classifications and good ones. To state that the goal of taxonomy is the expression of phylogenetic relationships is to belittle the true aims and importance of systematic zoölogy and has brought the whole science of taxonomy into an unfavorable light.

Is it any wonder that we still have whole phyla of uncertain systematic position when that position was supposed to be based on phylogeny? We can understand better now what G. K. Noble had in mind when he said that phylogenists and systematists sometimes appear to work on an instinctive basis, to "feel" their way to their systematic groupings. Can a definitive classification ever result from such methods of attack on the difficult and complex problems of animal relationship? We must have a more objective and more quantitative basis of systematic classification. Simpson and Roe (1939) have recently discussed methods by which morphological analysis may become more scientific, but there is also need of some additional data, of a new and independent and more objective basis from which to draw conclusions regarding the systematic position of animals. I refer to the use of biochemical analyses and especially to the serological reactions as evidence of biochemical similarity. But why all this bother about animal relationship? What is the importance of such knowledge anyway?

## VII. THE IMPORTANCE OF KNOWLEDGE OF ANIMAL RELATIONSHIP

Systematic serology, like systematic zoölogy, has its reason for being in its applications to problems of classification. The grand object of classification in any field of science is to group essentially like things together and to separate unlike things; and this grand object is essentially the same in biology as in geology or physics or chemistry. To be sure, many biologists have agreed that the goal of systematics or of taxonomy is the tracing of phylogenetic relationships, but this is certainly not "the truth, the whole truth, and nothing but the truth." Phylogeny—the tracing of the broad outlines of racial descent—is necessary to provide perspective for the understanding of evolutionary processes, but neither phylogenies nor genealogies are classifications. Even though complete pedigree records of all animal species were available, i.e., even with complete genealogical knowledge of species and individuals, we should still have no classification; the groupings, the separations between groups, would still have to be made; and when made *they should be based primarily on the natures of the objects grouped or classified*, not on the basis of what their remote ancestors may have been. Actually, phylogeny (in the absence of pedigree records we have no genealogies) is inferred from the character expression of existing and pre-existing organisms and can never be determined with absolute certainty. Even where the fossil records are most complete we are not justified in implying that the phylogeny has been determined with absolute certainty, however probable the phylogenetic conclusions may be in favorable material. The point is that, whether the phylogeny can be determined with reasonable certainty or not, we can and must *classify* organisms. Effective classifications of Protozoa, Porifera, Coelenterata, Ctenophora, Platyhelminthes, Nemathelminthes, Annelida, and other major groups of animals have been and are being made, though in none of these groups is the fossil record sufficiently complete to be decisive regarding the course of phylogeny. Of the two, therefore—phylogeny and classification—the latter is the more universal, objective, and significant in general biology.

Many zoölogists are ignorant of the real meaning of classification, of knowledge of animal relationship, and consequently they often work by trial and error in their investigations. Let us have clearly in mind what is implied in the term "animal relationship" before we set forth the evidence of the biological importance of knowledge regarding it. The term has had a variety of meanings, but as used here we imply by "animal relationship," "systematic relationship," i.e., knowledge of the position of a given species or larger taxonomic group with relation to other groups as determined by all the available pertinent comparative data regarding the essential natures of the organisms concerned. Fossil history, geographic distribution, genetic analysis, comparative morphology and embryology, comparative physiology, and biochemistry all provide data from which animal relationship in the meaning of systematic relationship may be determined. For most zoölogists the terms "systematic relationship" and "genetic relationship" are, or should be, synonymous; but let us have a more critical view of these terms. "Genetic relationship" as used in systematic biology implies common ancestry, but beyond this the term has no precise meaning, for there may be many kinds and degrees of genetic relationship. Thus we may have identical offspring whose genetic relationship must be considered very close. On the other hand, we may have amoeba and man whose genetic relationship, if there really is any, is very distant. Or we may have genetically related species and individuals differing in (1) genes, (2) gene arrangements, (3) chromosome

number, (4) cytoplasm, or in any possible combination of these differences. Since the term "genetic relationship" has therefore no precise meaning beyond that of common ancestry and since we cannot prove common ancestry for amoeba and man or other very diverse animals, we prefer to use the term "animal relationship" as above defined.

The primary duties of a scientific investigator, whatever be the field he tills, are these:

*a*) To learn the nature of the objects with which he deals
*b*) To classify these objects on the basis of the essential information gained by such studies, i.e., to group them according to their essential likenesses and differences
*c*) To find out how these objects have come to be what they are and to learn what determines their characteristics
*d*) To report to his colleagues from time to time regarding the results of his "observation and reflection," so that he may benefit by their criticism and they, in turn, may gain from any new knowledge and understanding thus made available.

Not one of these duties can be effectively performed without knowledge of animal relationship. For example, suppose we are investigating some phases of the nature of an organism whose systematic position is fairly well known. The first effective step is to make use of the organism's scientific name, which unlocks the storehouse of information already available regarding the nature of this organism and of its close relatives. If the organism is properly named, we can use this knowledge to guide us in (1) the verification of already reported observations and (2) the further experimentation designed to disclose new truth. Or suppose the species before us is new. There is no storehouse of knowledge available regarding this organism, and it might seem as though we were forced to "start from scratch." But this is far from true. An examination of the organism reveals characteristics which enable us to place it along with others essentially like it; and, whether the investigator himself or someone else succeeds in arriving at the proper systematic position of this type of organism, once this is known, the probable results of any experiment already applied to its relatives can be predicted, and the nature of the decisive experiments to be performed is thus indicated.

There may be approximately one million species of animals. Obviously, we cannot perform the same experiments on all of them. There is no real need for doing so, once we have a natural classification. Such a classification makes it possible to generalize effectively about related or essentially similar organisms and eliminates the need of countless repetitions of the same experiments. But clearly, if the experimental zoölogist is ignorant of the systematic position of his organism, he works in a very real sense by trial and error and lacks any basis for the determination of the importance of his findings, however accurate in detail they may be. Or if the systematist does his work badly and fails to gain groupings based on likenesses in essential traits, i.e., a truly natural classification, then scientific progress is impeded and investigation becomes ineffective.

Whether we study the present nature of organisms or seek to determine how they have come to be what they are, we need *systematic* knowledge. We cannot understand the nature of evolutionary processes without knowing what the results of evolution have been. The results of evolution in any line of ancestry are genetically *related* organisms with varying degrees of resemblance and difference; and knowledge of animal relationship, which is in this case based upon genetic relationship, is therefore indispensable to the student of evolution. In sum it can be said that a natural classification is necessary for the organization of our knowledge about animals and that therefore systematic zoöl-

ogy is one of the most fundamental divisions of biology. Furthermore, in proportion as systematic zoölogy is important, the potential value of systematic serology increases, for it is clear now that a natural system of classification requires more than a morphological basis. What, then, of the actual and potential contributions of systematic serology to the development of a definitive natural classification of animals?

## PART II. A SURVEY AND CRITIQUE OF REPRESENTATIVE STUDIES IN SYSTEMATIC SEROLOGY

Limitations of space compel a very condensed statement of these studies. As a matter of fact, it will soon become evident that most of the investigations in systematic serology have failed to provide comparable data because of inadequate techniques. Truly, it may be said, there has been all too little good serological research.

### I. THE FORMATIVE PERIOD, 1895–1910

The agglutinin reaction was recognized as a distinct phenomenon of immune serum by Bordet (1895) and was first thoroughly studied by Gruber and Durham (1896). The precipitin reaction was discovered by Kraus (1897). The technique used by Kraus was not adequately described. He apparently mixed various quantities of antiserum obtained from goats with the clear bacterial filtrates used for injection and observed a clouding followed by the development of flocculi, which eventually (within twenty-four hours at 37°) settled out under a clear supernatant fluid. This is the original illustration of a qualitative flocculation technique and involves varying, as well as unknown, amounts of the primary reagents. Kraus not only performed the homologous reactions but tested each of the antisera prepared (anticholera, antityphoid, antiplague) with all the antigens available, and only the homologous reactions were positive. As first described, therefore, the production of precipitates was *completely specific*.

The report of the occurrence of these specific precipitates with materials of bacterial origin was soon followed by researches concerning antigens of animal origin. Thus Tchistovitch (1899) described how he had injected rabbits, guinea pigs, dogs, and a horse with eel serum and found that the antisera thus obtained possessed both antitoxic and precipitating powers for eel serum. Tchistovitch also injected horse serum into rabbits and stated that the antihorse sera thus obtained were completely specific, in confirmation of the work of Kraus regarding the specificity of the reaction. The only evidence Tchistovitch mentions in support of this claim of complete specificity is his statement that antihorse sera did not react with ass serum, although giving abundant precipitate with horse serum! This astonishing result seems to indicate some lack of comparability in his homologous and heterologous tests for antihorse sera prepared in the way Tchistovitch describes (3 cc. serum given five or six times) are much more likely to be incapable of distinguishing horse and ass at all, even with much more adequate and sensitive methods. Probably Tchistovitch unwittingly used an excess of ass serum and thus prevented the heterologous precipitate from forming.

The foregoing researches provided no basis for the development of systematic serology by the use of precipitin reactions, for if all antisera react only with the homologous antigens there is no way of obtaining evidence of chemical or serological similarity, no indication of blood relationship. The first report which gave any hint that these precipitates could be useful in systematic zoölogy was that of Bordet (1899). The serum of a rabbit

repeatedly injected with fowl serum precipitated fowl serum *and also pigeon serum!* Again the details of technique are lacking, and we have only the first small step toward the goal of systematic serology. Another small step was taken by Bordet when he discovered that rabbits injected with guinea-pig serum failed to develop precipitins. This was an indication that an injected animal will not respond to the injections of proteins from closely related species, and this very inability to respond may serve as a guide to close relationships.

These and the remaining representative studies of the formative period in systematic serology may be briefly summed up in the following account. Bordet (1895) recognized the distinctness of the agglutination reaction. Gruber and Durham (1896) made a careful study of the agglutination reaction, found it not completely specific. Kraus (1897) discovered the precipitin reaction, claimed it to be completely specific as applied to bacterial filtrates. Bordet (1899) extended the precipitin reaction to antigens of animal origin; the reaction was found not completely specific (antifowl serum reacted with pigeon). Nolf (1900) separated horse serum into globulin and albumin fractions; he found these to be serologically distinct, and only the globulin was antigenic. Myers (1900) used crystallized hen egg albumin (method of Hopkins and Pinkus) and sheep and bullock globulins. Antihen egg albumin serum reacted with duck egg albumin also; antisheep serum globulin reacted less strongly with bullock serum globulin than with the homologous antigen, and the same was true of the reciprocal test. Apparently, Myers was first to use the term "precipitin." Uhlenhuth (1900) found that antihen egg white reacted also, though less strongly, with pigeon egg white. He used the antigen dilution method but not quantitatively. Landsteiner (1900, 1901) discovered three of the four principal human blood groups; O, A, and B. Von Decastello and Sturli (1902) discovered the fourth of the original human blood groups (AB). Stern (1901), Wassermann and Schütze (1901), Nuttall (1901*a*), found antihuman sera to react only with the bloods of other primates; they all viewed the reaction as diagnostic of blood stains in legal medicine. Nuttall (1901*b*, *c*, *d*) rapidly increased the knowledge of the results of testing antisera with many bloods; he found the intensity of the reaction to parallel the systematic position of the species tested; he emphasized the value of such knowledge to systematic zoölogy. More "powerful" antisera had the greatest range of reaction. Nuttall (1902) summarized the results on five hundred bloods, which confirmed previous reports, and described a new procedure for measuring the volume of precipitate. Linossier and Lemoine (1902*a*, *b*, *c*, *d*) used different proportions of antigen and antiserum and failed to get complete neutralization of antigen and antibody at any concentration. They found a wide range of reaction of their antisera, the relative intensities varying with systematic position, and concluded that the evolutionary transformation of species proteins must have been continuous. They reported that excess antigen inhibits formation of precipitate, that serum albumin was antigenic, and that precipitins were relatively heat stabile. Lamb (1902) used "sedimentation tubes" to measure the volumes of precipitate obtained with anticobra venom serum tested with various dilutions of the venoms of other snakes. The results appeared to parallel systematic positions of the species. Ascoli (1902*a*, *b*) developed the ring test and noted its more definite end point but made no titrations of related species. Ewing (1903) demonstrated the narrowing of the range of reaction of an antiserum following its dilution. Uhlenhuth (1903) found antiegg white sera to react in accord with systematic position of birds. He found antilens sera had broad ranges of reaction (no distinction among lenses of many mammals and even birds). Antimammalian

lens sera reacted less strongly with frog and viper lenses. He used lens solutions of approximately equal concentration as determined by nitric acid test. Von Dungern (1903*a*, *b*) studied carefully the reactions between antisera to mollusk and crustacean plasma. He found complete neutralization of antigen and antibody at certain proportions and proved that injection of a single antigen (haemocyanin) led to the production of more than one kind of antibody. He showed that a given quantity of antigen can combine with more antibody than is necessary to precipitate the antigen. Nuttall (1904) summarized the results of sixteen thousand precipitin tests, reviewed the literature to date, and concluded that in general the precipitin reaction gave data useful to taxonomy. Schur (1904) measured the volumes of precipitate in centrifuge tubes, using the antigen dilution method effectively. He obtained curves showing optimal proportions and pointed out the fundamental distinction between the "sensitivity" of an antiserum as determined by its titer and its actual antibody content. Unfortunately, he made no consistent application of this first "good" technique to systematic serology. Hamburger (1905), Hamburger and Arrhenius (1906), and Arrhenius (1907) used a technique essentially similar to that of Schur and measured the volumes of precipitate obtained with antisheep serum tested with various dilutions of sheep, goat, and bullock sera. These are the first data of systematic serology which have valid *quantitative* significance for animal relationship. The volumes of precipitate were greatest with sheep, less with goat, and least with bullock sera, in accord with the systematic relationship of these species. Friedenthal (1905) found no significant differences in reaction of different developmental stages of mice. He found that flightless birds reacted together and thus failed to support the view that they were morphologically convergent. He obtained weak reactions with the blood of the frozen Siberian mammoth tested with antiserum to Indian elephant. Welsh and Chapman (1906, 1910) used known weights of dried antigens and antisera in a comparison of egg whites of various birds. The tests paralleled the systematic position of these species. Fleischmann and Michaelis (1907) summarized the characteristics of the precipitin reaction in terms of optimal proportions. Franceschelli (1909) first used the Kjeldahl reaction to determine the nitrogen (and protein) content of the precipitates by difference. He injected rabbits with definite amounts of antigen per kilogram of body weight of the rabbit. Von Dungern and Hirszfeld (1910) proved the inheritance of the four human blood groups.

The essential knowledge relating to precipitins and agglutinins gained during this formative period may be put together as follows.

A. *Knowledge relating to the preparation and properties of antigens and antisera.*—Most proteins of animal origin are antigenic, the chief exceptions being proteins from a species very closely related to the injected animal. Such antigens may be preserved in fluid condition after sterilization or with the aid of antiseptics, in dried condition on filter paper, or as dried powder. Precipitating antisera are produced in rabbits following the repeated injection of sera or egg proteins, etc., at intervals of several days. The precipitins thus obtained are associated with the globulins of the antisera, and they are relatively heat stabile; they may be preserved sterile in the fluid state in the icebox or they may be temporarily preserved with the aid of antiseptics or they may be dried.

B. *Knowledge relating to the manner of performing the reaction.*—The precipitin reaction is best performed by using constant antiserum with a series of dilutions of antigen; the agglutinin reaction with constant antigen and increasing dilutions of antiserum. The precipitates obtained may be measured by weight, by volume, or by nitrogen determina-

tions. The sensitivity of an antiserum as indicated by its titer bears no simple relation to its content of antibody. Electrolytes are necessary in these reactions which are best performed at approximate neutrality, and the reactions are more rapid at 37°.5 C. than at lower temperatures.

C. *Knowledge relating to the nature of these reactions, especially the precipitin reaction.*—The reaction is a chemical one which may go on to completion where simple antigens and antibodies are used in optimum proportions. The effects of varying the proportion and concentration of reagents are as follows: With antigen constant, the amount of precipitate increases with increase of precipitin. With antiserum constant, the quantity of precipitate increases to a maximum and then decreases to zero with increase of antigen. The amount of precipitate decreases as the volume containing definite amounts of antigen and antibody increases. The composition of the precipitates obtained in homologous reactions varies, depending upon the proportion of reagents used. With any given antiserum the composition may be constant at optimal proportions, and the bulk of this precipitate comes from the antiserum.

D. *Knowledge relating to the systematic implications of these reactions.*—The injection of simple or complex antigens leads under suitable conditions to the production of antisera capable of reacting more strongly with homologous than with heterologous antigens. The injection of a simple antigen may lead to the production of more than one kind of antibody. Prolonged immunization generally increases the precipitating and agglutinating powers of antisera and to some extent their sensitivity and tends to broaden their range and make them less discriminating. The range of an antiserum, i.e., its capacity to include a variety of heterologous antigens, is decreased by dilution of the antiserum, and its discriminating capacity is correspondingly increased. In general the intensity of these serologic reactions runs parallel to the systematic positions of the species tested, and reciprocal tests commonly agree. In the case of the human blood groups—O, A, B, and AB—the capacity to produce the agglutinogens and agglutinins is determined by inheritance.

This summary is proof enough of substantial progress during the formative period of systematic serology. In fact, the rate of increase of knowledge in this field has never since been equaled. However, most of the researches in this period are no more than semiquantitative in nature. For example, the great bulk of Nuttall's sixteen thousand tests are described in qualitative terms and represent only crude estimates of the amounts of precipitate obtained from a single proportion of antigen and antibody taken from some unknown part of the curve of optimal proportions of antigen and antibody. Even when Nuttall measured the volume of precipitate with great care, as he did in his "quantitative" experiments, he still did not know where his selected proportion fell on the curve of optimal proportions, and consequently there is no proof that any of his measurements are comparable. Of those who measured the volumes or weights of precipitate, apparently only Schur and Hamburger and possibly Lamb have used a technique which could give comparable results. Of those who used the sensitivity method—employing ring test or flocculation test to determine the end points of the antisera titrated—apparently no one has satisfied the requirements for good serological research necessary to give comparable results. Much of the subsequent research in systematic serology was below the level of the best work of these early years, and this applies especially to the next score of years—the "Middle Ages" of systematic serology. Our comment on the researches of this next period in the history of systematic serology will be very condensed.

## II. THE "MIDDLE AGES," 1910–30

The researches in systematic serology published during this score of years represent the conflicting views of those who found promise for the future in the data they reported and those who obtained inconsistent results lacking any apparent relationship to problems of systematic zoölogy. On the credit side of the ledger of systematic serology the following reports are representative. Hektoen (1914, 1918*a*, *b*) reports upon the production of sensitive precipitating antisera in rabbits and fowls, the titrations being made with the ring test. Hektoen (1922, 1923), Hektoen and Schulhof (1922, 1923, 1924), Hektoen and Welker (1924, 1925), and Hektoen and Cole (1927, 1928) report upon the preparation and serological reactions of purified lens proteins, haemoglobins, serum proteins, and egg proteins. Obviously, such composite mixtures as egg whites and sera contain many distinct antigens, and their corresponding antisera are also composite. The antisera used were generally highly specific, better suited to the identification of these antigens than to their comparative serology; but the methods used are fundamental for the future progress of systematic serology in that they show the way to the acquisition and comparison of really corresponding single antigens and relatively simple antisera.

Bernstein (1925) discovered the correct mode of inheritance of the O, A, B, and AB blood groups as a system of triple alleles. Evans (1922) and Mason and Sanford (1924) recommend buffered salines for serological work. These are now widely used. Tsukasaki (1922) and Scimone and Torii (1923–24) report that extraction of antigens with lipoid solvents (alcohol, ether) increases the specificity of their corresponding antisera. Mollison (1924) made volumetric measurements of centrifuged precipitates from antiprimate sera reacted with primate sera. Taking only one point on the curve of reaction for his comparisons, Mollison found some disagreement in the reciprocal tests with sera of apes and monkeys. In general, the results did agree with systematic position, and the discrepancy among reciprocals was no greater than might be expected where mixed antigens of unknown protein concentration were used.

Boyden (1926) reinvestigated the relationships of common Mammalia using the ring test. Some of the results are reproduced in Figures 5, 6, 7. These figures show that the sensitivity method can give fairly consistent results when antigens are comparable in total protein and even though antisera are produced in both rabbits and fowls. In general, the reactions do parallel the systematic position of the species tested and support Nuttall's thesis that serological comparisons can be of assistance to the student of animal relationships.

Landsteiner (1928) called attention to the relation between the serological comparison of proteins and systematic zoölogy in the following words: "In the case of proteins the serological and chemical properties parallel closely the zoological relationship and the changes are gradual. . . . . Thus one could roughly construct the zoological tree merely on the basis of precipitin reactions." On the other hand, the agglutinin reaction with human red blood corpuscles showed markedly discontinuous evolution and sharp intraspecific distinctions.

Further evidences in support of Nuttall were provided in the work of Brown and Heffron (1928), Wilkoewitz and Ziegenspeck (1928), Sasaki (1928), Canning (1929), and others. Of these reports, that of Canning deserves special notice. When he used antisera prepared by the injection of extracts of particular organs and tissues of *Ascaris lumbricoides*, the various tissues of the same species showed some serological distinction.

Comparisons were also made with other species, and in general the results paralleled the systematic position of these organisms. One source of error in these comparisons was the procedure of making saline suspensions of the same amounts of dried-tissue powders, *without regard to the amount of antigen which actually went into solution.* The better procedure would have standardized the antigens on the basis of amount of antigen in solution, and until this is done we cannot be sure of the relative degrees of serological distinction among the various tissues and organs of *Ascaris.*

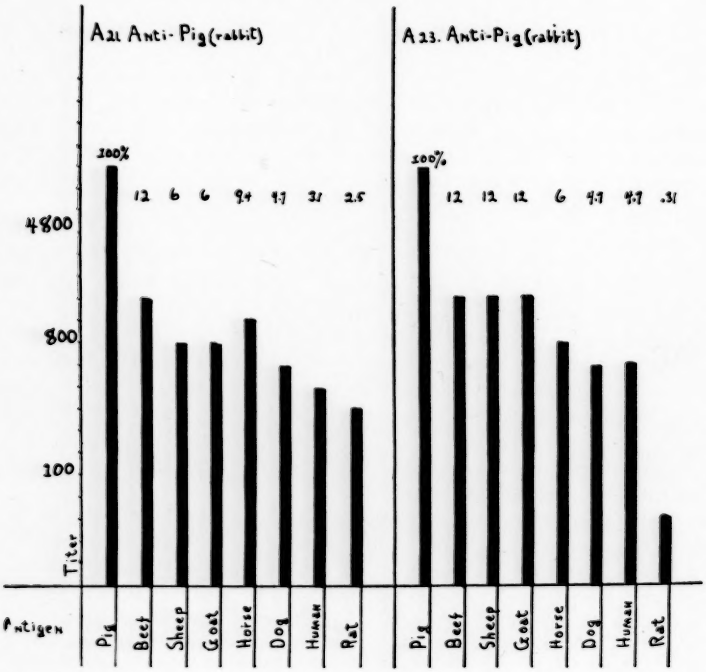


FIG. 5.—The results of titrating, by means of the ring test, two antipig sera having the same homologous titers, with a series of mammalian antigens (from Boyden, 1926).

The above reports all generally confirm Nuttall. In the meantime, a series of contradictory claims was made by Friedberger and Collier (1919), Friedberger and Jarre (1920), Friedberger and Meissner (1923), who emphasized the "heterogenetic" nature of precipitin reactions, i.e., their lack of parallelism to the systematic positions of the species tested. In these researches we need to bear in mind that the precipitin reaction was used in a relatively crude way, i.e., the original protein concentrations of the antigens were not determined, and, furthermore, the antigen dilution series had great gaps in it. Meissner (1923) reported that of seventy-eight different antisera over half (56 per cent) were highly specific, reacting only on the homologous or closely related heterologous antigens. The

remainder were less specific, and of these about one-fourth reacted equally with heterologous as with homologous blood. These latter antisera would be of little use to the student of animal relationship, but even these could usually be made discriminating by dilution. But why the bother about such "aspecific" antisera? The natural thing to do would be to discard the small proportion of antisera which cannot be used for relationship studies and to work with the majority which do give consistent results.

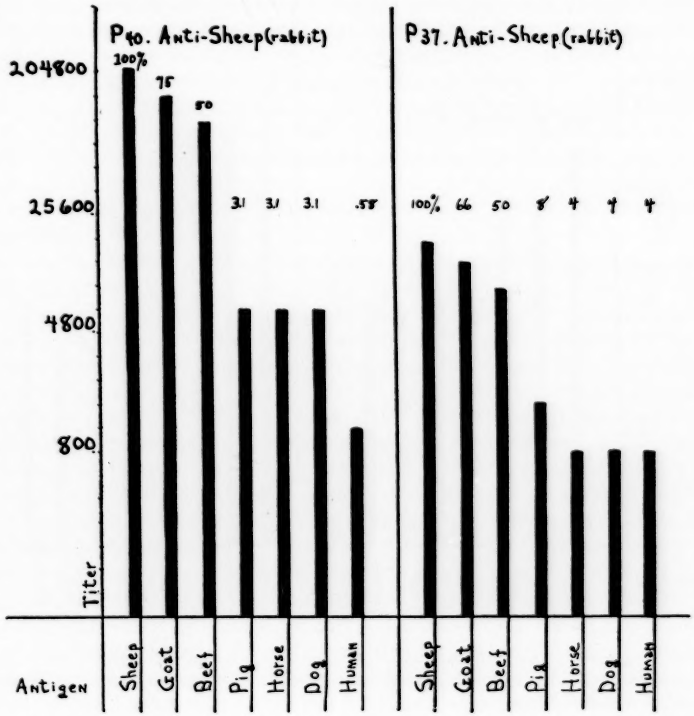


FIG. 6.—The results of titrating, by ring test, two antisheep sera of different sensitivity (from Boyden, 1926).

Finally, there are reports of Erhardt (1929*a*, *b*), which are in part summaries of preceding investigations and in part original researches. In these papers Erhardt tabulates the data of a great many other studies, but he puts them all on the same basis regardless of the techniques used and the sources of error involved. His own titrations with invertebrate antigens generally agree with systematic position; but, when nearly the whole of the results of investigations in systematic serology are before him, he is unduly impressed by the amount of inconsistency and disagreement, which should have been expected on the basis of the inadequate techniques of investigation used. Erhardt is correct in stating

that often we cannot be sure that the antigens are really comparable, for neither weight nor nitrogen content of the antigens will always guarantee serological equivalence. But he is not right in assuming that, whenever there is lack of agreement between the morphological and serological comparisons of species, the former must always be right and the latter wrong.

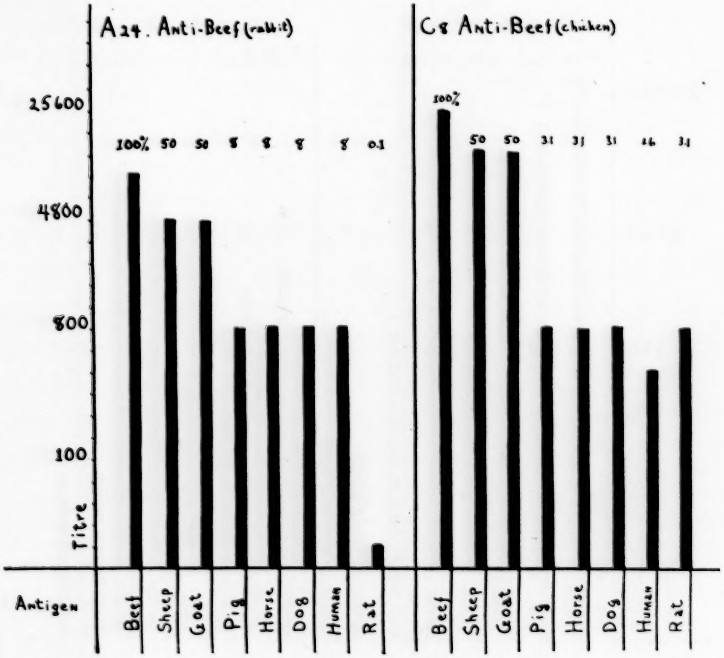


FIG. 7.—A comparison of the ring test results obtained by the use of two antibeef sera, one produced in a rabbit, the other in a fowl. Note the general agreement of these two antisera, except for the reactions against rat serum (from Boyden, 1926).

## III. RECENT TIMES, 1930–42

Without any clear line of demarcation between our chronological subdivisions of the developmental history of systematic serology we pass on to the gradual accumulation of a preponderance of evidence in favor of the use of the agglutinin and precipitin reactions as guides to systematic relationships in animals and through the work of Landsteiner (1936), Marrack (1938), and others to an increasing understanding of the conditions affecting serological specificity. Here follows a list of the representative works in this period with brief comments, those investigations which support Nuttall's thesis being given first.

Hektoen and Boor (1931) reported on antihaemoglobin precipitins. Most of their antisera give results in accord with systematic position. Wherever antisera react too broad-

ly (as, e.g., equally with mammalian and avian haemoglobins), their range can be narrowed and their systematic significance increased by simple dilution of the antiserum. Prolonged immunization broadened the range of these antisera, dilution transformed such antisera into useful systematic reagents.

Wolfe (1933, 1935, 1936, 1939*a*, *b*), Wolfe and Baier (1938), have presented a series of reports of considerable importance to students of systematic serology. The results of these researches dealing principally with common Mammalia generally accord with systematic position. In addition, it is clearly demonstrated that injection procedures affect the quality of the antisera produced and that, in order to get comparable results, similar injection procedures must be used. For discriminating, sensitive antisera of narrow range they used a short injection series. Reinjection of such rabbits with a similar injection series gave less discriminating and more broadly inclusive reagents which produced large volumes of precipitate. Reinjection of rabbits with minute amounts of antigen brought up the sensitivity to its former level, without altering the discriminating capacity of such antisera. These papers thus point the way to the regular preparation of antisera useful for systematic researches and thus constitute an important step forward along the way toward standard methods.

Boyden (1932) made a graphical expression of the relationships of common Mammalia, which required three dimensions. Boyden and Noble (1933) used the ring test in a study of common Amphibia. They standardized the antigens in terms of total nitrogen of sera and appeared to solve the old problem of the correct systematic position of *Siren* and *Necturus* (Fig. 8), which were found to be relatively distant from *Cryptobranchus*, a truly primitive species. The failure of *Siren* to metamorphose as other salamanders do can no longer be considered a primitive character. Boyden (1934) discussed the relative advantages and disadvantages of the serological and morphological approaches to systematic zoölogy and pointed to the complementary nature of the data of these two bases for the determination of animal relationships. A brief report by Boyden (1936) related the discovery of some convergent reactions with crustacean sera, which were eliminated by extraction with ethyl ether.

Makino (1934) found that precipitin reactions ran parallel to systematic position in mollusks, and Martin and Cotner (1934) found a general parallelism between precipitin reactions and systematic position of moths. Mollison (1934, 1936), Mollison and von Krogh (1937), and von Krogh (1937) studied primates, measuring the volumes of precipitate after centrifuging. In general their results accord with systematic position; but a certain amount of disagreement in reciprocals led them to the hypothesis that the evolution of proteins runs only in the direction of increasing size and complexity of their molecules with the accumulation of determinant groups. A primitive primate (such as *Macacus rhesus*) would have relatively more small-sized molecules in its serum with their primitive determinant groups, a more specialized primate (chimpanzee, man) would have relatively more large-sized molecules with their added specialized determinant groups. They submit this hypothesis to indirect tests, such as passage of the sera through filters under the assumption that relatively more of the larger molecules will be held back, and by measuring the volume of coagulated protein after the use of inorganic protein precipitants. Mollison's curves are of importance to systematists, whether his hypothesis of evolution of protein by increase in size of molecule is correct or not. As a matter of fact the results of the ultra-centrifuge studies of Svedberg and others do not support Mollison's theory, for there is no regular increase in the size of haemoglobin or haemocyanin molecules as we

go from primitive to specialized Vertebrata or Crustacea. Obviously these proteins, differing in their serological reactions, have evolved in other aspects than mere size, and this surely is what would be expected for serum proteins also. Interesting as Mollison's hypothesis is, it cannot be considered more than a hypothesis at present.

Eisenbrandt (1938) and Wilhelmi (1940*a*) investigated the reactions of extracts of helminths. The former used saline extracts of fresh material, and the results generally

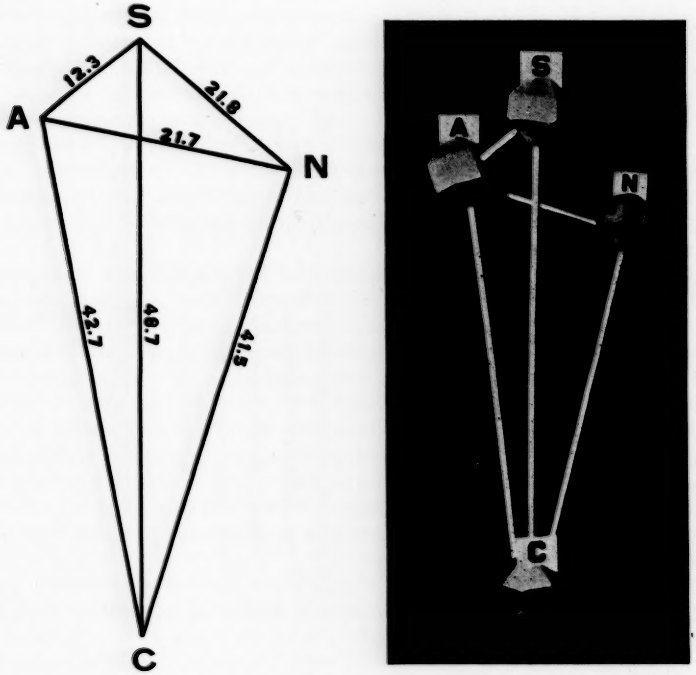


FIG. 8.—A diagram and model to show the interrelationship of *Cryptobranchus* (*C*), *Amphiuma* (*A*), *Siren* (*S*), and *Necturus* (*N*), based on ring tests with standard antigen dilutions. The relative "distances" are shown at the left (from Boyden and Noble, 1933).

agreed with systematic position. Perhaps the most interesting results were those indicating a closer relationship of the Acanthocephala to Platyhelminthes than to Nemathelminthes—results supported by some embryologic and anatomical considerations. The latter based his antigenic comparisons on the weight of dried, lipoid-extracted antigen actually in solution, and the results were remarkably consistent and significant to systematic zoölogy. Comparison of different ontogenetic stages revealed no serological differentiation. Levine and Moody (1939) studied rodent relationships by ring test, the results generally accorded with systematic position. Landsteiner and van der Scheer (1940) measured the volumes of precipitate obtained with antihen egg albumin sera tested with the egg albumins of common fowls. The mean values of a number of antiegg albumin

sera (twenty-two) were statistically significant, even though only one proportion of antigen and antiserum was used in each case, and these mean values were in accord with the systematic position of the species used. The above values were obtained by testing only a single antigen dilution and a single amount of antiserum and do not certainly represent, therefore, the results obtainable when the whole antigen titration curves are compared. The ring tests were much less discriminating, for several series of injections of the hen ovalbumin were made, and this, as is well known now, tends to make antisera about

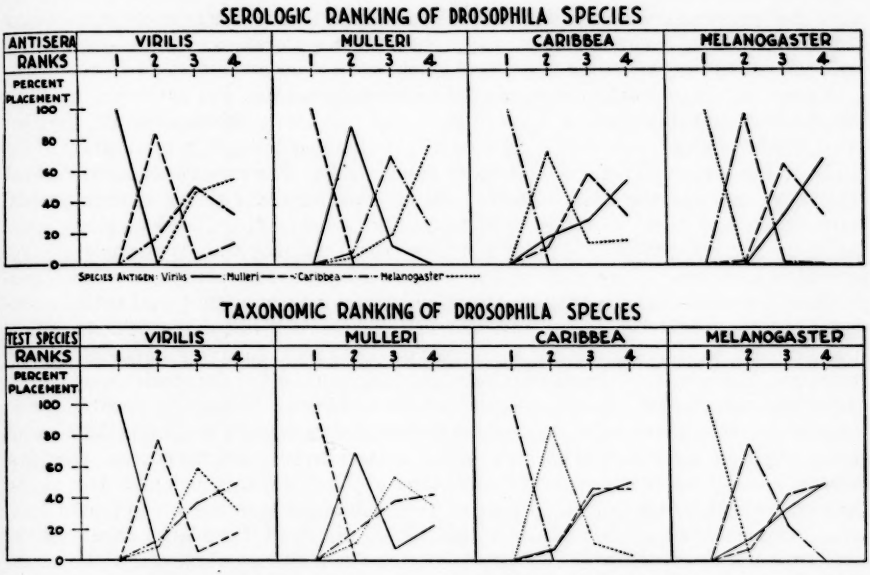


FIG. 9.—A comparison of the serologic ranking of *Drosophila* species with their taxonomic ranking based on fourteen morphological characters. Note the essential similarity of the corresponding curves for each antiserum and test species. Thus in the case of *D. virilis* antiserum and in the corresponding case where *D. virilis* is used as the standard for the taxonomic comparisons, the figure shows that *D. virilis* ranks first in 100 per cent of the cases and second in none of the cases. *D. mulleri* ranks first in no cases, second in about 80 per cent of the comparisons, third in about 5 per cent of the comparisons, etc. (from Cumley, 1940).

equally sensitive to similar antigens. The results with absorbed antisera indicated that the antisera produced in response to the injection of purified hen egg albumin contain multiple, qualitatively distinct antibodies. The view is taken that cross-reactions between proteins of kindred species are due generally to chemical similarity in determinant structures and not necessarily to the distribution of identical determinant groups among the related proteins.

Cumley (1940) summarized the results of several types of serological investigation and compared these estimates of the relative similarities of these species with their systematic positions as determined by fourteen morphological characteristics. The results are graphically shown in Figure 9, but Cumley's paper should be studied carefully by those inter-

ested in making a fair comparison of the results of comparative serological and morphological taxonomy. The results of this comparison were that the serological and morphological techniques always presented the same species for second rank, but they did not always agree in regard to the third and fourth positions. However, the serological tests could agree among themselves with regard to the third and fourth positions, whereas the morphological methods could not always be depended upon for that much agreement. As is well known, morphological taxonomy seldom gives us any quantitative interpretation of systematic positions, yet with morphological analysis far above its usual level of effectiveness the serological analysis was found to have the advantage. A recent abstract (Cumley, Irwin, and Cole, 1942) reports the segregation of serum-protein differences among progeny of species hybrids in doves.

A new tool useful in the comparison of precipitin reactions was developed by Raymond Libby and described in 1938 (Figs. 2 and 3). Using this instrument, Boyden (1939) and DeFalco (1941) briefly describe results obtained through its application to the study of the sera of Crustacea and birds, respectively. The serological comparison of crustacean sera has the advantage that the sera are relatively simple in their protein constitution and could therefore be serologically corresponding (see Fig. 4). According to the careful analysis of Allison and Cole (1940), haemocyanin is the only protein present in such sera. There could still be nonequivalence in such sera due to varied proportions of haemocyanins, all having the same relative copper content; and antisera produced in response to their injections could still vary in their range of specificity and in their proportions of different kinds of antibodies. The results so far obtained by this new technique, however, accord well with the systematic positions of the species tested when native sera are studied. In this comparison the turbidities developing over the whole range of the reactions of constant amounts of each antiserum with increasing dilutions of each antigen are summated in the form of the relative areas under the curves. Here is a more adequate basis of comparison than the use of the ring test, which uses data at the right end of such curves only, or the use of values obtained from the interaction of antiserum with some one of the reacting antigen dilutions. Even if the latter should be derived from the same parts of the curves, no single point is characteristic of a whole curve. This advantage of the use of the photronreflectometer in serological comparisons was emphasized by DeFalco (1941), who presented data from tests on the sera of common birds, which, again, generally confirm systematic relationships.

In the paper mentioned above (Boyden, 1939) reference is made to photronreflectometric comparisons of the sera of horse, mule, and ass. The curves are presented here for the first time (Figs. 10 and 11). The antisera used were unusually discriminating—ordinary antisera made in our regular way do not distinguish horse and ass so sharply and hence cannot be used to "put the mule in its place," so to speak. But, where the antisera are sufficiently discriminating, the results accord with the *known* genetic relationship of the mule and even the reciprocals show fair agreement. The point is made that, where antisera react in accord with genetic relationships where they are known, there is reason to expect that such antisera could be relied upon to give results of value to the systematic zoölogist even when species of relatively less certain systematic positions are being investigated.

The literature of agglutinin reactions has been collected by Snyder (1929), by Landsteiner (1936), and by Wiener (1939), hence there is no indication for an extended discussion in this place. That the distribution of isohaemagglutinogens and also that of iso-

haemagglutinins in the blood of primates is determined by inheritance is well known. Furthermore, the evidence that the capacity to develop agglutinogens of many kinds is inherited has been recently presented by Irwin, Cole, and others in species and hybrids of pigeons and doves (Irwin, Cole, and Gordon, 1936; Irwin and Cole, 1936*a* and *b*, 1937; Irwin, 1938, 1939; Irwin and Cumley, 1940; Cumley and Irwin, 1941*a* and *b*). In all this work we should say that the agglutinin reaction is, in a sense, more discriminating than the precipitin reaction, i.e., it can often sharply distinguish different individuals of the same species or even of the same genetic family. On the other hand, the distribution of agglutinogens among animals is less conservative than that of serum proteins, for, while

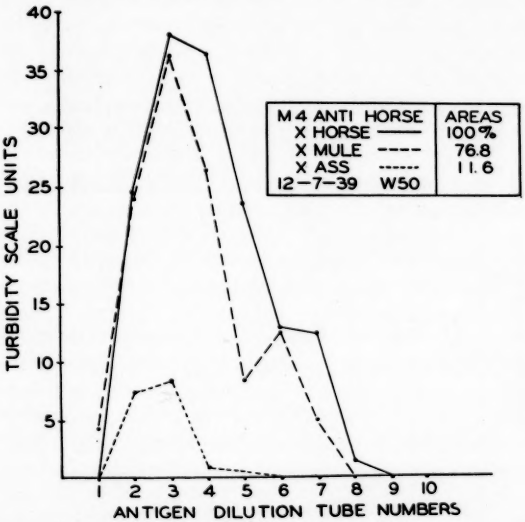


FIG. 10.—The results of titrating an antihorse serum with standard dilutions of the sera of horse, mule, and ass. Tube 1 in the standard series of antigen dilutions contains 1 part of protein in 500 parts of saline, and each succeeding tube or cell contains half the antigen of the preceding cell. The relative similarities of the sera are indicated by the areas under the curves (unpublished).

the sera of Primates and other orders of Mammalia are capable of being included within the range of precipitin antisera of sufficient strength and sensitivity, no one has yet found the *same* blood groups in different *orders* of mammals. From the standpoint of the interpretations of animal relationship we must point out that two humans belonging to the same blood group may have but a remote genealogical relationship and that their morphological and other physiological characters may be as different as are to be found within the human species. Hence the relative weight to be assigned to such a serological character as the blood group would be no more than that assigned to eye-color, which is not a safe guide to race either. In other words, these characters represent but a small part of the inherited constitution of the organisms compared—a part, furthermore, which is not definitely correlated with many other traits. On the other hand, the serological resemblance in serum or other proteins is more conservative and does show a high degree of cor-

relation with other characters of systematic value. Landsteiner (1928) has particularly called attention to this twofold system of species specificity in animals and concluded from it:

> Thus one could roughly construct the zoological tree merely on the basis of precipitin reactions. . . . . The specificity of cells is characterized on the contrary by the occurrence of so-called heterogenetic reactions which disclose the existence of similar structures in unrelated species. . . . .

There is some difficulty, then, in using these characteristics in the determination of animal relationship, and it seems, for example, impossible at present to decide which of the

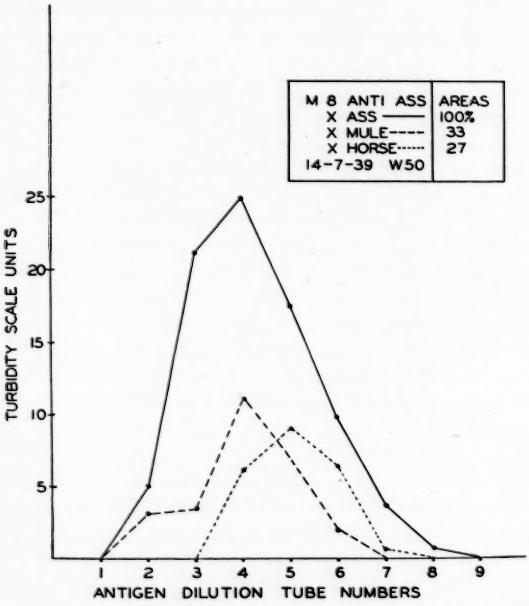


FIG. 11.—The results of titrating an antiass serum with the sera of ass, mule, and horse. These tests may be considered the reciprocal of those shown in Figure 10 (unpublished).

blood groups, O, A, or B, is the more primitive. The advantage of discriminating capacity as far as individuals alone are concerned *is* possessed by the agglutinin reaction, which thus can often distinguish *readily* among close relatives—a property which the precipitin reaction appears to lack.

In this last period of the history of systematic serology there are relatively few first-hand studies which contradict the general conclusion reached by the great majority of investigators that serological methods have considerable value for systematic zoölogy. There is a paper by Erhardt (1930), summarizing the results on birds. There are papers by Zuckerman (1934), and by Zuckerman and Sudermann (1935), both of which deal with Primates. Erhardt finds disagreement among the results of various serologists. He

seems to imply that serological results must agree, regardless of the techniques used in getting them, even with known sources of error left uncorrected. He takes the naïve position that whenever serological and morphological comparisons disagree only the latter can be right, forgetting two centuries of disagreements in regard to the systematic position of animals based on morphological comparisons. This attitude is incomprehensible to me.

Zuckerman (1934) discusses the primate affinities of the blood sera of lemurs. He finds that the lemurs present no evidence of a closer relationship to man, chimpanzee, or monkey than do nonprimate mammals such as cat, dog, horse, and ox. The possibility that lemurs may be convergent in their primate morphological expression is not considered. Mossman (1937) points out that there are both morphological and embryological characters which place the lemurs closer to the ungulate stock. In the light of these facts one can scarcely consider the primate relations of lemurs to be established beyond a reasonable doubt, and therefore criticism of serological results because they do not agree with a doubtful interpretation of systematic relationship is invalid.

Zuckerman and Sudermann (1935) report on "Serum Relationships within the Family Cercopithecidae," and even the title is incorrect, for the results of testing human chimpanzee, brown capuchin, and lemur sera are also included. The striking thing about this report is the inconsistency of some of the data obtained. For example, three antimacaque sera (*Macaca mulatta*) are tested with three or four macaque sera. One of these antisera (antimacaque *a*) reacts with relative titers as follows: with macaque *a*, 100 per cent; with macaque *b*, 43 per cent; with macaque *d*, 7.6 per cent. The same antiserum reacted also with human serum, 4 per cent; with chimpanzee serum, 42.5 per cent. These data are contrary to the results of experienced serologists in the following respects: (*a*) no one has ever succeeded in distinguishing by the ordinary ring test, with antigens comparable in total protein, the sera of different individuals of the same species. The fact is that the differentiation of even closely allied species is difficult with the ordinary ring test and can be done only with highly specific antisera made following short injection series or with previously absorbed antisera. (*b*) In the second place, sera which give evidence of being similar and scarcely distinguishable are treated similarly by *all* antisera in the experience of other investigators. But the tests of Zuckerman and Sudermann do not meet this specification, for human and chimpanzee sera, when measured by the antichimpanzee serum, are found to be very similar (100 and 89 per cent), but when viewed from the greater distance of two of the *Rhesus* macaque antisera they are found to be very different (4–42.5 or 9.5–49)! This result is against all serological perspective and the accumulated experience of many serologists. Interestingly enough, these three different macaque sera (*a*, *b*, *c*), found to be so different when tested with antimacaque *a*, were found to be remarkably similar when tested with several other primate antisera.

Much is made of the apparent lack of agreement of reciprocal tests, but the principle of reciprocal relationships was never meant to apply to inconsistent data. Actually half of the reciprocals did agree, which is surprising, considering the obvious limitations of the data. Zuckerman and Sudermann seem a bit confused regarding the meaning of their own data, for they claim that their results show the precipitin test to be incapable of distinguishing between different individuals of the same species, whereas the results I have quoted actually appear to do the reverse. What the reasons for these inconsistencies may be we cannot discover. The animals used were "dead to begin with," having been the subjects of various operational experiments. They may have been heavily drugged.

That they were normal representatives of their species in terms of serum proteins is not clear.

On the basis of such results the conclusion is reached that "the serum-precipitin test is of limited value in tracing phylogenetic relationships." On the basis of such results, *what* conclusion is valid? None, unless it be that precipitin testing must meet certain minimum requirements. Serological investigations must be continually improved, and no technique is final if known sources of error still remain uncorrected. We shall return to this point in the final discussion.

## PART III. GENERAL DISCUSSION

The representative results of serological investigations reviewed in this paper reveal that the data of systematic serology are fragmentary and sometimes apparently conflicting. The theory of systematic serology seems sound, the applications are incomplete and sometimes inconclusive. Yet in spite of the fact that known sources of error have not been completely eliminated and that comparisons have often been attempted where the techniques were really not comparable, the great majority of the data do appear to have significance for systematic zoölogy.

In the face of this situation what should be done? The "hit-and-run" investigator may quickly give up, concluding that serology must always remain subordinate to morphology as guide to animal relationship. Occasionally a serologist of considerable experience does this, unable as he appears to be to distinguish consistent from inconsistent data and relate them to the degree of adequacy of the techniques employed. We have had famous examples in recent times of eminent investigators who gave up too soon in the investigation of a particular problem.

The situation in systematic serology today is similar to that which held in systematic morphology two centuries ago. Fragmentary and conflicting data were available, and the very foundation of systematic zoölogy was insecure. As a matter of fact, it is still true that data of systematic morphology are incomplete and often in apparent conflict, but are we to conclude therefore that comparative morphology and taxonomy have no significance in general biology and should be given up? Certainly not. Systematic serology does not as yet give data indicating a simple solution to the problems of animal relationship. Neither does comparative morphology or phylogeny. There is no simple solution to the exceedingly complex biological problems of classification, but we need more investigations, not fewer, more data, not more gaps, in our knowledge. The problems of the determination of animal relationship must be solved; they are of fundamental importance.

Some of the data of systematic serology are inconsistent in themselves. Surely this should not be surprising, where such data have been obtained by methods which leave so many variables, so many sources of error, uncorrected. There are relatively few serological studies which have been carried out in comparable ways, and therefore disagreements in both qualitative and quantitative data are, under those circumstances, inevitable. Thus the use of antisera produced in different ways with consequent widely differing ranges and grades of specificity cannot produce comparable results. The failure to make antigens equivalent in at least their total protein content is a common source of error. The use of the ring test, which titrates the sensitivity of an antiserum, in comparison with the measurement of volume of precipitate obtained somewhere along the curve of

optimal proportions, is another illustration of the lack of truly comparable methods. The use of "aspecific" sera produced after the injection of large amounts of antigen or after a prolonged immunization period is no indication of the value of the results to be obtained, when useful antisera produced in the proper manner are tested upon a variety of serologically comparable antigens. I do not believe that a single one of the papers reviewed herein meets *all* the requirements for good serological research which we have set forth, yet the majority of them present data which have some significance for systematic zoölogy.

Some have criticized the results of systematic serology because, although consistent in themselves, they do not coincide with some particular hypothesis regarding phylogeny or some pet scheme of classification based on comparative morphology. With so many diverse notions regarding the phylogeny and classification of certain groups of animals it is asking too much that serology should agree with all of them, and of course it would be no help to systematic zoölogy if it did. There have been many occasions in the past where, if serology had agreed with the current view of animal relationship based on morphology, both would have been wrong. For example, when barnacles and tunicates were mollusks and *Limulus* was a crab, any criticism of serology because it failed to agree with those views would, of course, have been invalid. Fortunately, serological methods escaped being relegated to eternal subordination to systematic morphology at those times because serology was not then known. But the same type of criticism is being made today. If serological data are in clear conflict with genetic relationship where that is *known*, then and only then is such criticism valid.

Now there are many just criticisms of systematic serology, among them the following: (1) The investigator does not state, and apparently has not determined, the range of error of his own techniques. (2) The data are inconsistent, because noncomparable reagents or methods have been used, such as (*a*) antisera of different grades of specificity, (*b*) complex mixtures of antigens lacking even approximate serological equivalence, (*c*) crude estimations or even measurements of different characteristics of the serological reactions used, (*d*) failure to take advantage of the available knowledge and to improve the technique wherever possible. When all serological investigation meets the specifications of good serological research as outlined in this paper, then we shall move quickly toward our goal.

The principle of reciprocal relationship may or may not hold; as a matter of fact it has not as yet been given a fair trial. Some appear to believe that it means that rabbit A, injected with any amount of an unknown mixture of antigens, will yield an antiserum of the same range and grade of specificity as rabbit B, injected with another mixture and in another manner. This is simply absurd. But, when antigens and procedures are more nearly comparable, the majority of reciprocals do agree. But whether this is true or not there remain important advantages in the data of systematic serology. Thus, from the standpoint of objectivity alone, biochemical comparisons surpass most morphological comparisons. Furthermore, there are indications that the degree of similarity in serum proteins particularly may be decisive regarding the nature of the organism. Such proteins reach all parts of the organism and must be in harmony with all tissues. Sudden biochemical evolution in serum proteins would probably be lethal, i.e., the organisms' own antibody mechanism would immediately come into operation. As Landsteiner has frequently pointed out, the evolution of such proteins is gradual; they are conservative hereditary traits. If the weight assigned to some character of an organism by the sys-

tematic zoölogist is based upon (*a*) its conservatism, (*b*) its availability, (*c*) its objectivity, (*d*) its constancy during the life-cycle, (*e*) the readiness with which it may be subjected to quantitative description, then the serological comparison of the characteristic proteins of the animal body must stand high in his estimation.

The greatest apparent limitation of systematic serology is its restriction to existing species. To some this is a serious handicap. But must it be so considered? After all, there are about one million species of animals in existence, and there is plenty of work to be done to bring them into a convenient and effective classification. Besides this there may be primitive serological characters among existing animals. Just as the morphological expression of certain existing brachiopods goes back to the beginning of the Cambrian period, so serological characters, protein constitutions, may also be conservative. Granted that there are no fossil sera as there are fossil bones, conservatism is clearly indicated by a wide distribution of given characters in existing types. Thus the lens proteins of vertebrates are relatively conservative biochemical elements of the individuals possessing them, and serological perspective can be gained through their study, just as it can be observed in fossil series obtained from rocks laid down long ago.

Speaking of perspective in serological characters brings us to the consideration of what may be termed "serological dimensions." We refer to the quantitative estimates of degree of similarity in the sera of various animal groups. What do we learn from these estimates? In the first place, regarding the relative positions occupied by related species, we have found that the comparison of even small groups of animals requires three dimensions for its graphical expression (Figs. 8, 12). Both Darwin and Haeckel stated that family trees really required more than two dimensions for adequate graphical expression, but neither of them succeeded in making such trees. Fürbringer (1888) was apparently the first to do this, but his tree lacked any quantitative basis. The three-dimensioned figures referred to above were the first to have this quantitative and objective basis. Even three dimensions may not suffice to show all the taxonomic relations of a large number of species to one another, hence only small groups of species may be used at a time. But surely it is better to portray accurately the relative degrees of similarity to one another of small groups of species than to attempt to put them all into one expression in a crude and misleading way. In the second place, we have been shown that the birds as a group are relatively close to one another, much more so than mammals, and this agrees with our understanding of their systematic positions based on morphology. When our quantitative data are obtained with more nearly comparable antisera, then we may indeed be able to state that a certain degree of serological resemblance indicates a family or an order in mammals, another degree of resemblance an order or family of birds, etc. This would be helpful in assigning limits to the higher systematic categories, groups which are now often admittedly difficult to define. An interesting start in this direction has been made by Wilhelmi (1940*a*).

It is my hope that the difficulty of assigning an end point to homology may in time be overcome by serological methods. This brings us to the question of the extreme range of precipitin reactions. Experience with antiavian sera indicates that all the species of birds may be included within the range of powerful antisera. Such antisera may even include other classes of vertebrates, such as reptiles, and these antisera referred to are specific enough to have systematic values within the class of Aves. I do not wish to overemphasize the importance of these weak reactions which indicate the more distant relationships. They are subject to more error than usual simply because they are weak; and

the conditions under which the tests are made come to have an even greater importance than they ordinarily have. Because of this, the precipitin reaction should not be judged solely on the basis of such reactions as occur at the very limits of sensitivity of the antisera employed, as the chief critics of the value of this reaction as guide to animal relationships have tended to do. The extreme range of antisera tested against animal proteins extends beyond the limits of a single phylum. Such antisera, specific enough to be useful to the systematic zoölogist, have been described by Wilhelmi (1940*b*), who reports

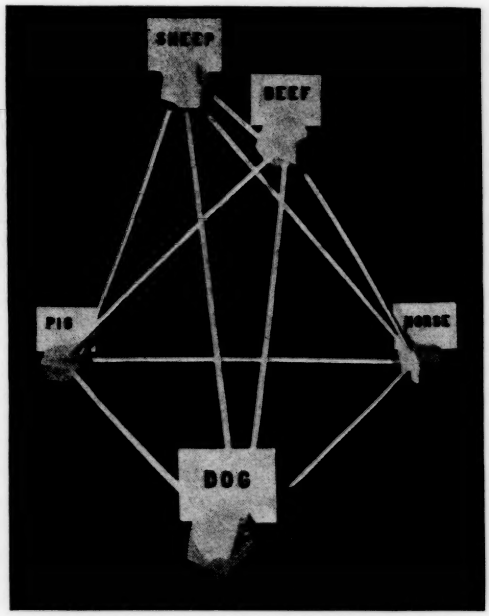


FIG. 12.—A model to show the relative positions and "distances" of common mammals from one another, based on ring tests. Each connecting rod represents the results of the reciprocal tests between the species occupying the loci at either end; and the closer the relative positions, the greater the similarity of the sera tested (from Boyden, 1934).

data suggestive of a closer relationship between Echinodermata and Prochordata than between the latter and Annelida or Arthropoda. If such results are consistently obtained, we may be able to come to some more definite conclusion as to the ancestry of Chordata than would be possible on the basis of morphology alone.

We have already given evidence that serological reactions are based on hereditary capacities for antigen or antibody production. There is another aspect of the relationship between systematic serology and genetics, which may prove to be unusually interesting to zoölogists. This is the relation between the degree of similarity in serum proteins and the limits of crossability of animals. We know already that animals of different species which can give offspring have very similar serum proteins. Thus bison and cattle,

horse and ass, are difficult to distinguish by precipitin tests, and they hybridize with the production of one generation of offspring at least. Such degrees of interfertility in animals are evidence of relatively close genetic relationship. As is well known, in birds, because of their relatively close genetic relationship, interspecific and even intergeneric crosses commonly show some degree of fertility. A recent study of Sokolovskaia (1936) is concerned with the relation between capacity to hybridize and precipitin reactions of certain ducks and geese. In most cases the serological data derived from the ring test coincided with the capacity to hybridize. Hydrids between Muscovy and common ducks gave reactions intermediate between those of the parents, but, when the antiserum was made to the hybrid sera, some new properties were found in the hybrids which were not present in either of the parents.

These latter results parallel those found by Cole and Irwin in the study, by means of the agglutinin reaction, of the corpuscles of species and hybrids of birds. The relatively simple genetic basis for the principal blood groups in primates has long been known. In fact, the genetic basis for the blood groups O, A, B, and AB—a system of triple alleles—is so simple that it constitutes too small a part of the genetic constitution of the individuals and species to have great weight in taxonomy. Thus it is still not easy to decide which of these blood groups is the more primitive, and which the more recent, evolutionary steps. And the steps, as Landsteiner has so well described, are relatively large and discontinuous. The more recent studies of animal relationship involving agglutination show, in addition to such discontinuous expressions, others of a more gradual type; and these new facts will contribute more and more to the analysis of blood relationship in the higher animals. There is every indication, however, that increasing knowledge of serum or other proteins characteristic of the individual and species provides a conservative indication of the nature of the organism and must therefore be given a high value in the "analysis of racial descent."

The bulk of the evidence available indicates that serological characteristics are relatively constant during the life-cycle. In the case of the human blood groups, for example, the group to which an individual belongs is expressed early in ontogenetic development and stays the same throughout life. In the case of the serum or tissue proteins the same constancy has been noted. In other words, the genetic constitution of the individual regarding serological characters is expressed early and undergoes relatively little change. This brings out an interesting problem regarding the relation between serological constitution and morphological expression. It was stated by Sachs long ago that the form of an organ is determined by its chemical constitution. This was even referred to by J. Loeb as "Sachs's law," but actually it has never been proved or disproved. In a sense it is supported by all our genetic knowledge, for instance, the form of the wing of *Drosophila* depends on the interaction of one hundred or more genetic factors. But the real problem is this: How does the chemical composition of the wing differ from that of other parts of the body of the same individual? Canning found evidence of some degree of serological differentiation in the tissues of *Ascaris*. Wilhelmi finds no serological differentiation in the whole-worm extracts of some ontogenetic stages of helminths, but this is not conclusive evidence against Sachs's principle, for the antigens actually titrated by Wilhelmi may have been relatively inert protein or carbohydrate materials. Dr. Mabel Boyden and I have traced lens proteins into the chick embryo as far as the seventy-hour stage, but the lens forms some twenty or more hours earlier, and so the decisive question—Does

the morphological differentiation of the lens depend upon the chemical differentiation of lens proteins?—still remains unanswered. Whatever may be the final outcome of investigation to determine the relation between serological constitution and morphological expression, the relative constancy of the serological expression of the individual in its life-cycle has great advantages for the systematic zoölogist.

What of the future? Surely this is no time to quit! In fact, systematic serology has just begun. Systematic morphology might have been advised to quit in 1742, as it had failed to gain consistent results to that time, but fortunately, there were scholars who kept on, and systematic zoölogy in that stage of development is comparable to systematic serology now. Comparative morphology has roughly a two-hundred-year start over comparative serology in terms of actual and potential contributions to systematic zoölogy. This has its advantages and disadvantages. The great advantage is that critical comparative morphology has provided a solid basis with which to compare the results of comparative serology, and serological methods which fail to yield results in agreement with genetic relationship where that is fairly well known can be quickly discarded. The great disadvantage is that the new method is unfamiliar to many systematists and is taught by few who are competent to do so, with consequent relatively slow progress. We need help in solving problems, such as the following, and their solution will guarantee that the goal of systematic serology will soon be reached:

1. What constitutes serological equivalence?
2. What are the limits of individual variability in serum proteins?
3. What is the genetic mechanism responsible for the expression and persistence of serological characters such as the serum proteins?
4. What is the relation between serological constitution and morphological expression?
5. Is there serological convergence?
6. What direct criteria of serological primitiveness can be ascertained?
7. Can precipitin tests aid in solving the interrelationship of the phyla of uncertain systematic position?
8. Can systematic serology aid in reaching a definitive natural classification of animals?

As biological problems go, nowadays, these are important and interesting. As Nuttall did forty years ago, we again call the attention of zoölogists to them in the hope of gaining their active support.

## PART IV. SUMMARY

In the forty-odd years of its existence investigators in the field of systematic serology have discovered the following facts and principles.

1. Serological reactions may be used to indicate the biochemical constitution of organisms.

2. The antigenic constitution of organisms in general parallels the systematic position of the same organisms based on critical morphological study.

With regard to the relative importance of morphological and serological data in determining the systematic position of animals we must bear in mind:

3. That comparative morphology has roughly a two-hundred-year start over comparative serology and that the present available data in the two sectors of biology is no indication of the final contribution of either.

4. That comparative serology, like comparative morphology, is no simple guide to animal relationship. The very complexity of the problem demands the use of all possible

pertinent data. The data of systematic serology, where comparable methods are used, are as valid as those of systematic morphology, and the two methods of analysis should be considered complementary to each other.

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# NUTRITIONAL AND SUPPLEMENTARY FACTORS IN THE GROWTH OF CARNIVOROUS CILIATES[1]

(Two figures)

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DURING the past two decades a number of ciliated protozoa have been successfully established in bacteria-free cultures. The growth of certain holotrichous ciliates in sterile peptone solutions has been reported by Lwoff (1924), Taylor, Thomas, and Brown (1933), Hetherington (1933), Elliott (1933), Glaser and Coria (1935), and Johnson (1935). Although there has been considerable confusion concerning the taxonomic position of the various strains used by different investigators, the recent work of Furgason (1940) indicates that all these may be included in the single species, *Tetrahymena geleii* Furgason. The species described from this laboratory as *Glaucoma vorax* (Kidder, Lilly, and Claff, 1940) is now thought to belong in the genus *Tetrahymena*. Two species of other genera, *G. scintillans* and *Colpidium campylum*, have also been established in bacteria-free culture in this laboratory (Kidder, 1941*a*).

Three species of *Paramecium* have been reported as capable of growth in sterile media: *P. bursaria* by Loefer (1934) and *P. caudatum* and *P. multimicronucleatum* by Glaser and Coria (1933). A recent report by Taylor and van Wagtendonk (1941) indicates that at least one species of *Colpoda* can be cultured in a sterile medium containing yeast extract and a plasmoptysate of a marine variety of *Pseudomonas fluorescens*.

For the culture of all other ciliates it is at present necessary to introduce some other living organism as food. Such two-organism cultures, while less completely controlled than the pure cultures described above, do permit physiological studies that would have little or no significance with the older methods. Of course, aseptic technique to prevent outside contamination is just as necessary as in work with pure cultures. With the exception of the work of Brown (1940) and of Dewey and Kidder (1940) two-organism systems that have been described in the literature have involved as the food organisms pure cultures of yeast, bacteria, or algae. The availability of pure cultures of ciliate protozoa now has made possible a bacteria-free, two-organism system in which these saprozoic ciliates serve as food for larger carnivorous ciliates. This type of culture allows a more complete quantitative and qualitative control of food. With omnivorous ciliates, such as bacteria-feeders which are facultatively carnivorous, a comparison may be made between different types of diets and their physiological effects on the organism. As a technical advantage this method of culture furnishes large numbers of bacteria-free organisms that could otherwise be obtained only in small numbers by laborious sterilization techniques. In some cases merely allowing the supply of food ciliates to become exhausted furnishes a pure culture of carnivores. In any case, sterile carnivores may easily be separated from food organisms by simple washing techniques.

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Up to the present only a few species of carnivorous ciliates have been established in bacteria-free culture on a diet of other ciliates. It was found in this laboratory (Kidder, Lilly, and Claff, 1940) that *T.* (*Glaucoma*) *vorax* could be maintained in the carnivorous form indefinitely by supplying sterile *T. geleii* as food. Similarly, Brown (1940) compared the growth curves of *Leucophrys patula* and *T. geleii* when the former was feeding on the latter. Concerning well-known strictly obligate carnivores, there have been no reports of absolutely bacteria-free cultures. *Didinium nasutum* (Beers, 1933) and *Woodruffia metabolica* (Johnson and Evans, 1939) have been cultured on washed *Paramecium*, but in both cases the washing was insufficient to remove all bacteria.

The availability of *Tetrahymena* as a source of bacteria-free food for ciliates has not been followed by the adoption of this technique by many workers. Giese and Alden (1938), in an experiment incident to their work on cannibalism, washed *Stylonychia curvata* bacteria-free and supplied them with a pure culture of *Tetrahymena* in lettuce infusion. Whether or not these cultures of *Stylonychia* were maintained bacteria-free for considerable lengths of time was not stated. It seems probable, however, that these cultures were quite comparable to those of *S. pustulata*, to be described later. Their choice of lettuce infusion as a medium appears to have been particularly fortunate, since, in view of findings reported here, this undoubtedly supplied the supplementary growth factor without which a truly bacteria-free culture would have failed.

The present work is concerned with the nutrition of two species of hypotrichous ciliates, *S. pustulata* and *Pleurotricha lanceolata*, which are usually bacteria-feeders in nature but are facultatively carnivorous. Several strains of these organisms have been established in bacteria-free culture by supplying as food *Tetrahymena* from pure cultures. It has been found that such cultures are successful only if a supplementary growth factor is supplied in the medium. The investigation of the occurrence, nature, and properties of this growth factor comprises the major part of this work. A comparative study has also been made of the nutritive value of a number of microörganisms as food for these species.

## Material and Methods

### Carnivorous hypotrichous ciliates

Five strains of *S. pustulata* (Fig. 1, *A*) and four strains of *Pl. lanceolata* (Fig. 1, *B*) have been used in this work. Strain I of *S. pustulata* was originally obtained from a fresh-water pond at Woods Hole, Massachusetts. All other strains used were obtained from fresh-water sources in the vicinity of Providence, Rhode Island. Up to the present no single strain has been maintained in bacteria-free culture more than a year. Since both species are very common in nature, it was found less difficult to obtain new strains than to attempt to re-establish failing cultures. The cause of the loss of the strains used in the earlier parts of the work will be discussed later.

### Food organisms

Four strains of *T. geleii* were used in this work. Three of these were obtained by Professor G. W. Kidder: the H strain (formerly called *C. campylum*) from Dr. Robert H. Hall (1938), the E strain (formerly called *C. striatum*) from Dr. A. M. Elliott (1933), and the P strain (formerly called *G. pyriformis*) from Dr. Austin Phelps (1936). The fourth strain, W, was established in bacteria-free culture by C. L. Claff (1940). Three other species were also used in some experiments. These were *T. vorax* (Kidder, Lilly, and

Claff, 1940) and *G. scintillans* and *C. campylum* (Kidder, 1941*a*). The flagellates used were *Astasia klebsii*, obtained from Dr. Herman von Dach (1940), and *Chilomonas paramecium* and *Euglena gracilis*, sterilized and established in culture in this laboratory (Kidder, 1941*b*). The strain of yeast, *Saccharomyces cerevisiae*, was isolated from a cake of bakers' yeast (Fleischmann). *Stichococcus bacillaris* was obtained from Dr. T. M. Sonneborn and *Chlorella* sp. from Dr. J. B. Loefer (1936).

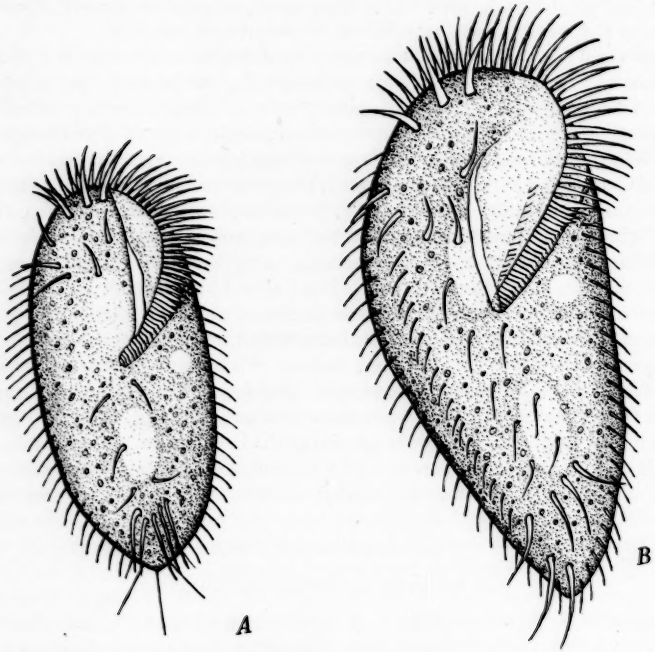


FIG. 1.—Drawing of carnivorous hypotrichs from life. ×500. *A*, *S. pustulata*; *B*, *Pl. lanceolata*

All the organisms of the genus *Tetrahymena* were cultured on agar slants containing 2 per cent proteose-peptone and 0.5 per cent dextrose. A thin film of moisture in which the ciliates could spread over the surface was found to facilitate growth. The slants were incubated 2 or 3 days at 26° C. and then kept at room temperature. A rich, spreading, creamy growth of ciliates developed at the lower part of the slant. For best results these cultures were used within a week after inoculation. The same agar medium was found convenient for the cultivation of *Euglena*, *Stichococcus*, *Chlorella*, and yeast. It was also used for routine contamination tests described later. The use of this solid medium for the culture of food organisms greatly simplified the method of supplying food, since large numbers of organisms were thus obtained with a minimum of transfer of the medium by scraping them off the surface with a bacteriological loop. *Glaucoma scintillans* and *C.*

*campylum* were cultured in the fluid medium recommended for these organisms (Kidder, 1941*a*).

### STERILIZATION

The method employed for the sterilization of the carnivorous ciliates was a modification of that used by Parpart (1928). In this modification Syracuse watch glasses inclosed in Cellophane bags were substituted for the Petri dishes used in the original method (Kidder, Lilly, and Claff, 1940). A number of other ciliates in addition to these hypotrichs have been washed bacteria-free by this technique, which has now become standard practice in this laboratory for sterilizing larger organisms.

### CULTURE

For routine culture of the carnivorous ciliates ordinary bacteriological test tubes (15×125 mm.) containing about 7 ml. of medium were used. The medium at first consisted of a suspension of autoclaved yeast cells prepared by washing the growth from a 48-hour proteose-peptone-dextrose agar slant in 200 ml. of distilled water. Later a more convenient source of yeast cells was found in the preparation known as Brewers' Yeast—Harris (Kidder, Lilly, and Claff, 1940). A stock solution was prepared by boiling 5 gm. of this powder in a liter of Pyrex-distilled water for 15 minues. The bulk of the residue of the yeast cells was removed by filtering successively through cheesecloth, cotton, and finally several times through filter paper. This stock solution was autoclaved in 100 ml. quantities and stored until needed. For the culture medium some of this stock solution was diluted to ten times its volume with distilled water, tubed, and autoclaved.

The food organisms were introduced into the culture tube just before inoculating the carnivorous ciliates. For routine cultures usually a loopful of the H strain of *T. geleii* was taken from an agar slant. The carnivorous ciliates were at first introduced with sterile pipettes or by aseptically pouring in a small amount of fluid from a flourishing culture. Both these methods were later superseded by the use of a special bacteriological loop which was modified to give a greater volume by forming from the wire a helical coil of three turns. The volume of medium (0.01 ml.) transferred by this instrument was usually sufficient to introduce several organisms from a thriving culture. Transfers with this wire helix had the same advantage as those with a wire loop, and the increase of the inoculum of the old medium was negligible in relation to the volume of the new medium.

In the case of *S. pustulata* the routine cultures usually reached a maximum concentration in about a week and were subcultured at that time. *Pleurotricha lanceolata* attained a maximum concentration in a somewhat shorter time, usually in about 5 days. In the case of the latter species the food ciliates were usually completely exhausted at this time, and within a few days the carnivores died so rapidly that after 10 days subcultures were often impossible. With *Stylonychia*, on the other hand, very often a few scattered food ciliates remained, but these were too few to maintain the carnivorous forms at a maximum concentration. Instead of dying rapidly, as did *Pleurotricha*, these organisms showed considerable resistance. Small starving *Stylonychia* could be found in cultures a month after inoculation. These small forms, however, were very difficult to re-establish on the carnivorous diet, and cultures much over 2 weeks old were usually discarded.

Tests for bacteriological sterility were an important part of the cultural work. Immediately after each strain was established on the carnivorous diet its sterility was estab-

lished by tests with liquid and solid nutrient media of several kinds. To guard against the possibility of the presence of organisms which would not develop in such media, tests were also made with a synthetic medium of the following composition:

| | Grams | | Grams |
|---|---|---|---|
| Asparagin | 2.0 | $MgSO_4 \cdot 7H_2O$ | 0.4 |
| Dextrose | 5.0 | NaCl | 0.1 |
| $(NH_4)_2SO_4$ | 0.5 | $CaCl_2$ | 0.1 |
| $K_2HPO_4$ | 0.5 | $FeCl_3$ | 0.02 |

Distilled water....1,000 ml.

(Adjusted to pH 7.4 with N/1 NaOH solution)

Once the original sterility of a culture was determined, it was necessary to make further routine tests periodically as a precaution against unsuspected contamination. These tests were performed by streaking about 0.01 ml. of the culture medium on the surface of an agar slant and incubating at 26° C. for a week. Growth of *Tetrahymena* on this medium could readily be distinguished from bacterial growth, so that little difficulty was experienced in detecting contaminants.

## The Role of Living Food Organisms

### *Tetrahymena geleii*

*The phases of growth.*—In the standard medium (supplying the necessary supplementary growth factor) excellent growth of both *Stylonychia* and *Pl. lanceolata* was obtained with all four strains of *T. geleii* available in this laboratory. While no significant differences were observed in the results obtained with the different strains, the H strain (formerly called *C. campylum*) was used for most of the experimental work, as well as for routine stock cultures. The course of the cultures of both carnivorous hypotrichs was found to follow the phases of growth described for other ciliates (Phelps, 1936; Dewey and Kidder, 1940; Kidder, 1941*a*, 1941*c*).

*a*) The lag phase: A lag phase was observed which in part was comparable to the lag phase of the saprozoic ciliates, such as *Tetrahymena*, since it was negligible with ciliates taken from the phase of logarithmic growth of the previous culture and prolonged with ciliates taken from older cultures. An additional factor was found to affect these facultative carnivores. Small organisms often had difficulty in ingesting the relatively large food ciliates. In most cases the first division did not occur until after about 24 hours, but subsequent divisions then occurred at intervals of from 8 to 12 hours. The lag in such cases was the total time required for the small organism to begin feeding on *Tetrahymena*, to increase its size to that typical of carnivorous forms, and finally to undergo the preparatory changes antecedent to division. A similar relationship between the size of the organism and the length of the lag period has been noted by Dewey and Kidder (1940).

*b*) The phase of logarithmic growth: After the initial lag period the reproductive rate rose to about three divisions per day and remained at that level as long as the *Tetrahymena* were present in sufficient numbers to provide food. In the case of *Pleurotricha* the logarithmic growth rate continued after all food ciliates were removed from the culture. There was some evidence that this rate was maintained for one or two divisions after the exhaustion of the food, since individuals transferred to sterile distilled water at this time continued to divide at a high rate. A similar maintenance of a high division rate after the exhaustion of food has been described in the case of *Perispira* feeding on *Euglena*

(Dewey and Kidder, 1940). Apparently a sufficient reserve of food is built up on the carnivorous diet to permit division at the maximum rate. The opaque granules in the cytoplasm appeared to be concerned in this storage of food, since they gradually disappeared after the exhaustion of food. In cultures of *Stylonychia* the first part of the phase of logarithmic growth was very much the same; but, as the food organisms were reduced to numbers about equal to the number of carnivores, some of the latter were apparently no longer able to secure food and began to revert to the small size and transparency characteristic of starving organisms.

*c*) The stationary phase at maximum concentration: There was a marked difference between the two species of hypotrichs in the length of this period. In *Pleurotricha* the stationary phase was very short, usually not more than a day in duration. In *Stylonychia* the stationary period was always more prolonged, and the presence of a few food organisms was often a complicating factor. Although some strains of *Stylonychia* had a division rate nearly equal to that of *Pleurotricha*, the consumption of food ciliates per organism was lower, and the supply was not reduced as quickly. The maximum concentration, therefore, was reached a day or two later than in *Pleurotricha*.

There was some evidence, however, that another factor, perhaps concerned with the products of metabolism, might be operative at the time of maximum concentration. Further addition of food ciliates to cultures at this time did not lead to a resumption of the logarithmic growth rate. On the contrary, the population of carnivores remained practically constant, and large numbers of *Tetrahymena* were still present several days afterward.

*d*) The phase of death: In the case of *Pleurotricha* the decrease in population was extremely rapid. The total absence of food was, of course, an important factor, but the failure of the organisms to undergo appreciable reduction in size seemed to preclude the possibilitity that dying organisms had completely exhausted protoplasmic food reserves. *Stylonychia*, on the other hand, was more resistant; and active individuals, often extremely small and transparent, were observed in cultures over a month old.

*Growth in flask cultures.*—Although the different growth phases were quite apparent in the course of cultures maintained in test tubes, they were more conveniently studied quantitatively in cultures of larger volume. The method used was essentially that described by Kidder (1941*a*, 1941*c*) in his growth studies on holotrichous ciliates. Each large culture flask containing 500 ml. of medium was inoculated with 10 ml. of a heavy suspension of *T. geleii* and a known number of carnivorous hypotrichs (usually 15 or 20). The suspension of food ciliates was prepared by washing off the entire growth from an agar slant. Immediately after inoculation a sample was drawn from the flask to determine the population of food ciliates at the start of the experiment. The populations of both food organisms and carnivores were then determined daily. The data so obtained in one of these experiments were used to plot the growth curves shown in Figure 2. The growth of *Tetrahymena* in the standard medium used in this experiment reached a maximum concentration of about 10,000 organisms per milliliter. As the carnivorous hypotrichs increased in numbers, this population was sharply reduced. The maximum concentration of hypotrichs in these cultures was about 200 organisms per milliliter, but higher concentrations (over 300 per milliliter) have been obtained in tube cultures when the food organisms were supplied in higher concentrations. In these experiments the population of food ciliates was reduced to less than 10 organisms per milliliter, but a few survived throughout the rise and fall of the population of hypotrichs. In the case of the

tube cultures of *Pleurotricha* the food ciliates were often completely removed, but this was rarely the case with tube cultures of *Stylonychia*. Otherwise, however, the growth of both species of hypotrichs in these flasks appeared to be quite comparable to that observed in routine culture in tubes. The growth curves shown in Figure 2, therefore, constitute a graphic representation of the principal phases of growth in cultures of these hypotrichs in standard medium and on a diet of *T. geleii*.

*Encystment and conjugation.*—Encystment was observed in all strains of *S. pustulata* and *Pl. lanceolata* when cultured in media containing bacteria. On the bacteria-free ciliate diet, however, the strains of *S. pustulata* and strain I of *Pl. lanceolata* were never observed to encyst. On the other hand, encystment was observed in strains II and III of *Pl. lanceolata* in cultures which were shown by tests to be bacteria-free. Conjugation was

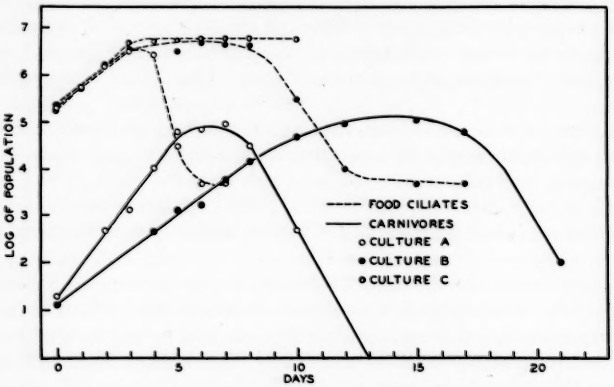


FIG. 2.—The growth of carnivorous ciliates in standard yeast medium. Culture A, *Pl. lanceolata* and *T. geleii;* culture B, *S. pustulata* and *T. geleii;* and culture C, *T. geleii* (control). The population data were based on the volume of the entire culture, i.e., five hundred times the population per milliliter. The temperature was 26° C.

observed in strains III and IV of *S. pustulata*, where it often occurred in bacteria-free cultures after the stationary phase had been reached. Exconjugants from these cultures isolated for the purpose of establishing new pure lines failed to resume the carnivorous diet, possibly because of their small size.

*Cannibalism.*—All strains of *S. pustulata* and strain II of *Pl. lanceolata* were found to become cannibalistic under certain conditions. Results of observations on cannibalistic "giants" of both species were similar to those reported by Giese and Alden (1938). Such forms were never seen, however, before the stationary phase at maximum concentration. Cannibalistic *Pleurotricha*, when introduced into cultures of *Stylonychia*, were never observed to feed on these smaller organisms, in spite of the close physiological and morphological similarity of the two species of hypotrichs.

## OTHER FOOD ORGANISMS

*Tetrahymena vorax* and *C. campylum* were found to support the growth of both *Stylonychia* and *Pleurotricha*. No important qualitative differences were observed in these cultures when compared with those in which *T. geleii* was supplied as the food or-

ganism. *Glaucoma scintillans*, on the other hand, did not support the growth of either *Stylonychia* or *Pleurotricha*. Examination of carnivores supplied with *G. scintillans* in hanging-drop cultures showed that this species was never ingested, although *Tetrahymena* supplied in comparable cultures was readily taken in. In a similar experiment *C. steinii* cultured on a single strain of bacteria, *Aerobacter cloacae* (Kidder and Stuart, 1939), was not ingested by either of these carnivorous species.

*Euglena gracilis* and *A. klebsii*, supplied in the standard yeast medium, supported growth of both hypotrichs. Successful cultures, however, were established only when the proportion of food organisms to hypotrichs was kept within certain limits. The proper balance between the two populations was especially difficult to maintain in the case of *S. pustulata*. Although *Stylonychia* grew when fed *E. gracilis*, the number of food organisms also continued to increase and was never reduced below the original inoculum. Cultures of *Pl. lanceolata* on *E. gracilis* were more satisfactory, since trophic food organisms were often almost completely removed. Nonmotile forms of *E. gracilis* which were numerous in old cultures were apparently never ingested. The complicated factors discussed by Dewey and Kidder (1940) in their work on *Perispira* fed on *Euglena* seem to be operative in the case of these hypotrichous ciliates. Flagellates as a source of food for these organisms, therefore, were definitely inferior to ciliate food when reproducible results under standardized conditions were required. Nevertheless, a possible advantage was seen in the longevity of cultures of *Pleurotricha* feeding on *Euglena*. These cultures were maintained at least three times as long after inoculation as when ciliates were provided as food. *Chilomonas paramecium* proved inadequate for the growth of either *Pleurotricha* or *Stylonychia*, but in this case the organisms were ingested—at least for a short time.

A suspension of living yeast (*Sa. cerevisiae*) was early found to support growth of *S. pustulata*. Yeast did not support as high a division rate or maximum concentration of *Stylonychia* as did the carnivorous diet, but growth was continued over a longer period. There was also remarkable longevity in these cultures after the stationary period had been reached. *Pleurotricha*, on the other hand, showed no growth in such suspensions of yeast cells. This constitutes the only important qualitative nutritional difference yet found between these closely related species of hypotrichs.

*Stichococcus bacillaris* and a species of *Chlorella* originally associated with *P. bursaria* were supplied in suspensions in the standard yeast medium. These organisms were ingested readily by the hypotrichs but failed to support growth beyond the first transplant.

During the course of this work a number of attempts were made to culture these hypotrichs by supplying a single strain of bacteria as food. Samples from cultures of hypotrichs in hay infusion, in which a mixed flora of bacteria provided an adequate diet, were inoculated on nutrient agar, and a number of different species of bacteria were thus obtained in pure culture. No single strain of those tested supported growth of the hypotrich either in hay infusion or in distilled water, although in a few cases there was abundant growth of the hypotrichs in the first transplant. In no case, however, was such growth maintained through several transplants. In routine cultures (containing *Tetrahymena* in standard medium) bacterial contaminations were found to affect the carnivores in different ways. Sometimes there was abundant growth of the hypotrichs, often continuing over a long period after the ciliate food was exhausted. Contaminants having this effect, however, did not support growth through several transplants in standard medium without the ciliate food. In other cases contaminants supported excessive growth of the food ciliates, but the hypotrichs failed to reach their usual concentrations. Still other con-

taminants had the effect of inhibiting the growth of both ciliates. This was usually the effect of rapidly growing types, but it was also found in the case of a few slow-growing bacteria which did not increase the turbidity of the medium. Finally, the most insidious type of contamination, which made periodic routine contamination tests necessary, had little apparent effect on the cultures for long periods. In such cases the presence of bacteria was detected only by the development of colonies on the agar medium used in the routine test.

### LIVING ORGANISMS AS A NUTRITIONAL NECESSITY

Relatively few species of ciliates have been found capable of utilizing killed food organisms. The culture of *Colpoda* in the absence of living organisms by Taylor and van Wagtendonk (1941) suggests the possibility of obtaining by special methods extremely labile substances which may be a growth requirement for some species. The previous work of Glaser and Coria (1933) in culturing *Paramecium* may also be given a similar interpretation. One of the principal reasons advanced for the nutritional differences between living protoplasm and dead material has been that the drastic treatment necessary to kill the organism has been sufficient to alter the protein constituents of protoplasm. As long as these experiments were confined to such microörganisms as bacteria and yeasts, it seemed probable that the proteins were denatured by the killing agent. With the use of ciliates as food organisms, however, it became possible to use much less drastic methods of killing.

*Tetrahymena geleii* may be killed by several different methods which would not suffice to kill all the population of a culture of yeast or bacteria. Temperatures of 50° C. or over are usually required to kill bacteria and yeast. A suspension of *Tetrahymena*, on the other hand, was killed in about 15 minutes at 37° C. after having been previously kept at room temperature. The rapidity of the temperature change was found important, since the ciliates were able to become acclimatized to this temperature if given sufficient time. After cooling, the tubes containing the dead ciliates were inoculated with *Pleurotricha* or *Stylonychia*. In some cases these cultures were transferred to Syracuse watch glasses in Cellophane bags for closer observation of the carnivorous ciliates. The organisms were never observed to ingest the killed ciliates with which they were surrounded, and there was no growth in any of these cultures.

*Tetrahymena* may also be killed by freezing, another method inapplicable to yeast and bacteria because of the survival of the resistant minority of the population. Not only were the *Tetrahymena* killed by the freezing, but in many cases the cells were broken up into small particles. No ingestion of this material by the hypotrichs was observed, and no growth occurred.

A third method of killing *Tetrahymena* is by sudden changes in the osmotic pressure of the medium. Several loopfuls of ciliates were suspended in 5 ml. of sterile distilled water and allowed to stand about 15 minutes. Then about 3 ml. of sterile 5 per cent sodium chloride solution was added, which promptly killed all the ciliates. The dead organisms were then washed by centrifugation with sterile distilled water to remove the salt and resuspended in standard medium in tubes. *Pleurotricha* and *Stylonychia* inoculated into these tubes were not observed to ingest the dead ciliates. There was no growth in any of these cultures.

The three methods of killing ciliates reported here are such that alterations of proteins in the protoplasm would not be expected as a direct result of the treatment. Of

course, this does not exclude the possibility of alteration of the chemical constituents of the protoplasm by some autolytic process as an indirect result of the treatment. The failure of the hypotrichous ciliates to ingest the dead organisms still leaves unsettled the question of whether this material would be nutritive if ingested. At present, material that is not ingested may be, for practical purposes, considered nonnutritive, and it is in this sense that the term has been used here.

In addition to these experiments with killed ciliates, suspensions of yeast were killed by heating tubes in a water bath at 60° C. for 3 days and then inoculated with *Stylonychia*. Yeast cells were also killed with toluene and then thoroughly washed in sterile distilled water. These killed yeast suspensions failed to support growth of *Stylonychia*. Similar experiments were not tried with *Pleurotricha* because, as mentioned previously, even live yeast is nonnutritive for this species.

## The Supplementary Growth Factor

### Natural occurrence

In the first attempts at culturing *S. pustulata* on bacteria-free ciliates it was discovered that, although *Tetrahymena* taken from pure cultures and supplied to nonsterile *Stylonychia* often supported excellent growth, when the same diet was supplied to sterile *Stylonychia* in distilled water growth never occurred. At this time attempts were also being made to culture the sterile hypotrichs on dead yeast, liver extract, and other media. Therefore, combinations of suspensions of living ciliates in various media were tried. The combination of sterile ciliates with autoclaved yeast cells in distilled water supported excellent growth of bacteria-free *Stylonychia*, and the first cultures of strain I were thus established. The medium first adopted for routine use was a suspension of autoclaved yeast cells. It was possible to use commercial pressed yeast cakes of bakers' yeast (Fleischmann's) as a source of yeast cells, but the amount of extraneous material was found to be a disadvantage. A better source of yeast cells was obtained by isolating a strain of yeast and culturing it on proteose-peptone-dextrose agar slants. In an autoclaved suspension the yeast cells settled out to the bottom of the tube, leaving a fairly clear medium in which growth could readily be observed. Strain I of *Stylonychia* was cultured in this medium. Later, for the other strains, the standard medium was developed, using Brewers' Yeast—Harris.

The yeast preparations have proved to be the most reliable source of the supplementary growth factor required by *Stylonychia* and *Pleurotricha* (Lilly, 1940). However, several media made from plant materials have been used successfully. Infusions of hay and rice polishings in distilled water were found to furnish enough of the supplementary growth factor to permit growth of both *Stylonychia* and *Pleurotricha* on *Tetrahymena* through several transplants. The growth rate was usually lower in these media; but, since the maximum concentration depends on the quantity of ciliate food available, the total number of individuals produced in these cultures was quite comparable to those in standard media.

In contrast to the growth obtained with *Tetrahymena* in these media, suspensions of ciliates in distilled water never supported growth of either species. The inoculated hypotrichs sometimes divided several times, probably on stored food reserves, and frequently were visible in the medium 2 or 3 days later. In these cases, however, ingestion of the food ciliates was not continued, and growth beyond the two or three "carry-over" divi-

sions never occurred. A number of different kinds of media tested also gave similar results to those obtained with distilled water. Such results were taken as evidence of the lack of the supplementary growth factor.

In testing media for the presence of the supplementary growth factor a routine method was used, based on the technique of culturing in standard medium. The tubes of media

TABLE 1

A COMPARISON OF THE SUPPLEMENTARY GROWTH FACTOR IN VARIOUS MEDIA

| Medium | Growth of Carnivores | Growth of Food Ciliates | Probable Content of Growth Factor |
|---|---|---|---|
| Autoclaved yeast cells | +++ | + | +++ |
| Standard yeast medium (Brewers' Yeast—Harris) | +++ | + | +++ |
| Particle-free standard yeast medium | +++ | + | +++ |
| Standard yeast medium with thiamin destroyed | +++ | − | +++ |
| Bacto-Yeast Extract, 1 per cent | − | +++ | − |
| Bacto-Yeast Extract, 0.1 per cent | − | ++ | − |
| Lettuce infusion | ++ | + | ++ |
| Hay infusion | ++ | − | ++ |
| Rice-bran infusion | + | − | + |
| Oat infusion | + | − | + |
| Kelp infusion | + | − | + |
| *Elodea* infusion | + | − | + |
| Spinach infusion | + | − | + |
| Bacto-Liver infusion | − | +++ | − |
| Bacto-Liver infusion, diluted to 10 per cent | − | ++ | − |
| Bacto-Liver infusion with thiamin destroyed | − | − | − |
| Difco Beef Extract, 0.025 and 0.0025 per cent | − | − | − |
| Difco Proteose-Peptone, 1 per cent | − | +++ | − |
| Difco Proteose-Peptone, 0.1 per cent | − | ++ | − |
| Bacto-Beef Blood, 0.1 per cent | − | + | − |
| Dilute rabbit blood, 1 per cent in distilled water | − | + | − |
| *i*-inositol, 0.1 per cent | − | − | − |
| Biotin solution, containing 0.1 γ per milliliter | − | − | − |
| Calcium pantothenate solution, 0.1 γ per milliliter | − | − | − |
| Vitamin B complex—solution of thiamin, riboflavin, nicotinic acid, and pyridoxin, 10 γ per milliliter of each | − | − | − |
| Mixture of inositol, biotin, pantothenate, and vitamin B complex | − | − | − |
| Balanced salt solution (Barker and Taylor, 1931) | − | − | − |
| Distilled water | − | − | − |

to be tested were each inoculated with a loopful of *T. geleii* (H. strain) from an agar slant. At the same time, control tubes of standard medium were prepared. All tubes were then inoculated with *Stylonychia* or *Pleurotricha* from a culture which had just reached the end of logarithmic growth. Each tube was examined after inoculation to make certain that active organisms had been transferred. The tubes were then incubated at 26° C. and examined daily for growth, using the control tubes of standard medium for comparison. Growth equal in *rate* to that in the control tubes of standard medium was recorded as 3 plus (+++). Growth which resulted in the consumption of practically all the food ciliates and the attainment of a *maximum concentration* comparable to the control tubes was

recorded as 2 plus (++) or 1 plus (+), depending on the difference in the growth rate. A slight increase in the number of inoculated hypotrichs without consumption of most of the food ciliates was considered as negative (−). Survival of the hypotrichs for 24 hours or longer in any medium was taken as sufficient evidence that the medium was nontoxic. Table 1 summarizes the results of these routine tests.

### THE ISOLATION METHOD OF TESTING

While the routine method was found satisfactory for testing media, owing to the "all or none" growth characteristics of the carnivorous hypotrichs, for certain phases of the investigation a more quantitative method of comparing growth in various experimental solutions was desirable. The method developed is a modification of the old isolation-culture technique (Calkins, 1933) to permit the maintenance of bacteria-free conditions throughout the experiment. Each culture was started with a single hypotrich isolated in a small drop of water in a serological tube. This was followed by the addition of 1 ml. of a suspension of food ciliates in distilled water and 1 ml. of the experimental solution. Growth in these cultures was followed by daily examination and by counting the number of individuals in each tube. Ordinarily five isolation cultures were used for each experimental solution. Each tube, after filling, contained one large carnivorous hypotrich, with the equivalent of about 0.03 ml. of the old culture medium, 1 ml. of experimental fluid, and 1 ml. of a suspension of *Tetrahymena* of known concentration. These tubes were incubated at 26° C. and examined daily for growth. Each tube was held so that it was properly illuminated by transmitted light and shaken to dislodge sediment and ciliates from the bottom. The large hypotrichs were easily visible to the naked eye under these conditions, and it was usually possible to count them without trouble. The tube was then checked under the dissecting microscope. The condition of the organisms was observed; and, if necessary, the count was rechecked under magnification until at least two counts in agreement had been made. This counting method, although admittedly only approximate, gave results that were within the limits of experimental error. When over 50 individuals were counted, a larger percentage of error was introduced, on account of the increased likelihood of counting the same individual twice as it changed position in the tube. The numbers of 100 or over recorded are only estimates as indicated by the plus sign (+). In the tables which follow, therefore, it will be noted that growth rate is more significant than the absolute numbers. The first 3 days after inoculation also are relatively more significant than the fourth or fifth. The tables do not include the observations made on the condition of the organisms, but in most cases where there was no growth the size and appearance of the organisms were also affected.

### THE EFFECTIVE CONCENTRATION

The various media containing the supplementary growth factor were effective only in relatively high concentrations. This was early indicated by the fact that relatively large inocula (up to as much as 10 per cent) of old media into distilled water still did not permit growth of the hypotrichs on ciliate food. The results of an experiment on the effect of diluting the standard medium are shown in Table 2. As shown in the table, dilution of the standard medium to 25 per cent was sufficient to show an effect on the growth of *S. pustulata*. Dilutions below 5 per cent are practically equivalent to distilled water, as far as their content of the supplementary growth factor is concerned. This experiment seems

to indicate either that the active substance, if it is a single substance, is required in relatively large amounts or that it is contained in its natural sources only in very small amounts.

### A COMPARISON WITH THE VITAMIN B COMPLEX

The occurrence of the supplementary growth factor in largest amounts in brewers' yeast suggested its possible identity with one or a combination of the known vitamins of the B complex, in which brewers' yeast is especially rich. At the time when this work was started, the following vitamin preparations were available: thiamin hydrochloride (vitamin $B_1$), riboflavin (vitamin $B_2$), nicotinic acid, and crystalline Factor 1 (vitamin $B_6$).[3]

TABLE 2

THE EFFECT OF DILUTION OF STANDARD YEAST MEDIUM

Numbers indicate the number of hypotrichs in each tube. At the start of the experiment each tube contained: 1 *S. pustulata* (strain II); 1 ml. of a dilution of standard medium; 1 ml. of distilled water containing approximately 1,000 *T. geleii* (H strain).

| Dilution of Medium in Tubes | Tube | Days after Inoculation | | | | | Dilution of Medium in Tubes | Tube | Days after Inoculation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | | | 1 | 2 | 3 | 4 | 5 |
| 50 per cent | A | 1 | 4 | 30 | 100+ | 300+ | 5 per cent | A | 1 | 1 | 4 | 3 | 3 |
| | B | 2 | 3 | 32 | 100+ | 300+ | | B | 1 | 2 | 7 | 7 | 5 |
| | C | 3 | 7 | 65 | 125+ | 300+ | | C | 1 | 3 | 7 | 7 | 6 |
| | D | 2 | 3 | 24 | 80+ | 200+ | | D | 1 | 1 | 2 | 4 | 7 |
| | E | 2 | 3 | 29 | 90+ | 200+ | | E | 4 | 6 | 12 | 11 | 11 |
| 25 per cent | A | 2 | 4 | 23 | 43 | 60 | 2.5 per cent | A | 1 | 2 | 2 | 2 | 2 |
| | B | 2 | 5 | 16 | 25 | 32 | | B | 1 | 1 | 2 | 2 | 1 |
| | C | 2 | 7 | 20 | 36 | 70+ | | C | 2 | 3 | 3 | 3 | 3 |
| | D | 3 | 11 | 51 | 85+ | 200+ | | D | 2 | 3 | 3 | 2 | 2 |
| | E | 1 | 2 | 3 | 3 | 2* | | E | 1 | 3 | 10 | 14 | 12 |
| 12.5 per cent | A | 1 | 11 | 32 | 44 | 64 | 1 per cent | A | 1 | 2 | 2 | 2 | 2 |
| | B | 2 | 3 | 12 | 16 | 12 | | B | 4 | 5 | 5 | 4 | 3 |
| | C | 1 | 2 | 2 | 2 | 2 | | C | 1 | 1 | 2 | 1 | 1 |
| | D | 1 | 3 | 8 | 11 | 14 | | D | 1 | 2 | 2 | 3 | 3 |
| | E | 1 | 3 | 5 | 3 | 3 | | E | 1 | 2 | 3 | 3 | 2 |

* Contaminated.

These substances were tested separately and in combination, using the isolation method. The concentrations used were in all cases higher than the minimum dosage required by ordinary laboratory test animals. In the case of thiamin and nicotinic acid toxic effects were observed in concentrations of 0.005 per cent, most of the animals failing to survive 24 hours. In the concentrations finally used in these experiments, however, the survival of practically all organisms for several days was taken as evidence of nontoxicity. Tables 3 and 4 summarize the results. In the case of *Pleurotricha* organisms isolated in vitamin solutions often underwent four or more divisions before growth finally stopped. These "carry-over" divisions proved difficult to control; but in no case did the growth in such cultures approach that in the control cultures in standard medium, in which growth

[3] The crystalline Factor 1 was obtained through the courtesy of Dr. S. Lepkovsky.

always continued until all the food organisms were consumed. With *Stylonychia* there were fewer divisions in the vitamin solutions, but the control cultures showed excellent growth. While the tables indicate merely the number of individuals in each tube on each successive day, it should be pointed out that there was also a marked difference in the appearance of the animals in the vitamin solutions, as compared with those in the controls. In most cases these organisms were of the same size as starving individuals in standard medium but differed from them in having opaque and granular cytoplasm. These qualitative differences have, however, proved difficult to use comparatively; hence, the number of organisms per tube has been selected as the sole basis for comparison.

TABLE 3

COMPARISON OF THE VITAMIN B COMPLEX WITH THE SUPPLEMENTARY GROWTH FACTOR

Numbers indicate the number of hypotrichs in each tube. At the start of the experiment each tube contained: 1 *Pl. lanceolata* (strain II); 1 ml. experimental solution; 1 ml. distilled water containing approximately 1,000 *T. geleii* (H strain).

| Experimental Solution in Tubes | Tube | Days after Inoculation | | | | Experimental Solution in Tubes | Tube | Days after Inoculation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | | | 1 | 2 | 3 | 4 |
| 0.001 per cent thiamin (vitamin $B_1$) | A | 3 | 5 | 11 | 23 | 0.001 per cent nicotinic acid | A | 2 | 3 | 3 | 5 |
| | B | 2 | 2 | 7 | 19 | | B | 2 | 2 | 2 | 3 |
| | C | 2 | 3 | 15 | 32 | | C | 4 | 11 | 28 | 32 |
| | D | 2 | 4 | 5 | 6 | | D | 2 | 2 | 2 | 2 |
| | E | 2 | 4 | 19 | 25 | | E | 1 | 1 | 1 | 2 |
| 0.001 per cent riboflavin (vitamin $B_2$) | A | 2 | 5 | 12 | 27 | Mixture of equal parts of the solutions of thiamin, riboflavin, Factor 1, and nicotinic acid | A | 2 | 3 | 5 | 14 |
| | A | 2 | 3 | 3 | 4 | | B | 3 | 7 | 11 | 22 |
| | C | 2 | 6 | 11 | 29 | | C | 2 | 2 | 3 | 4 |
| | D | 3 | 7 | 9 | 13 | | D | 2 | 4 | 10 | 17 |
| | E | 2 | 4 | 6 | 11 | | E | 2 | 3 | 5 | 12 |
| 0.00025 per cent Factor 1 (vitamin $B_6$) | A | 3 | 6 | 9 | 19 | Standard yeast medium | A | 7 | 26 | 70+ | 200+ |
| | B | 2 | 2 | 4 | 6 | | B | 6 | 28 | 75+ | 200+ |
| | C | 3 | 4 | 6 | 9 | | C | 4 | 22 | 60+ | 150+ |
| | D | 3 | 7 | 10 | 35 | | D | 5 | 18 | 55 | 125+ |
| | E | 1 | 1 | 1 | 2 | | E | 6 | 30 | 90+ | 200+ |

In addition to the experiments summarized in the tables, similar tests were made with the same vitamin preparations using the E strain of *T. geleii* as the food organism. Strain I of *Pl. lanceolata* was also used in some experiments. In all cases the general results were the same; there was no maintained growth in any of these pure vitamin preparations or in all of them combined. Hence, the supplementary growth factor required by *Stylonychia* and *Pleurotricha* in addition to ciliate food is not identical with thiamin, riboflavin, nicotinic acid, Factor 1 of Lepkovsky or a combination of these vitamins. Several of the "Bios" factors—inositol, biotin, and pantothenic acid—have been tested by the routine method, using each separately and also in combinations, including all the synthetic vitamins of the B complex. No growth-promoting effects were obtained with any of the combinations tested. These results have been included in Table 1.

These experiments, of course, do not prove that these vitamins are not required by the carnivorous hypotrichs, but only that they cannot be substituted for the supplementary growth factor. One of the vitamins of the B complex, thiamin, has been shown by Lwoff and Lwoff (1938) to be important in the growth of the food ciliates. Recently Elliott (1939) has published more detailed experiments, indicating the effect of thiamin in the nutrition of one of these ciliates (E strain).

Since the food ciliates themselves utilize thiamin in their own nutrition, it appears quite probable that they furnish this vitamin in the nutrition of the carnivorous forms

TABLE 4

COMPARISON OF THE VITAMIN B COMPLEX WITH THE SUPPLEMENTARY GROWTH FACTOR

Numbers indicate the number of hypotrichs in each tube. At the start of the experiment each tube contained: 1 *S. pustulata* (strain II); 1 ml. experimental solution; 1 ml. distilled water containing approximately 1,000 *T. geleii* (P strain).

| Experimental Solution in Tubes | Tube | Days after Inoculation | | | | | Experimental Solution in Tubes | Tube | Days after Inoculation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | | | 1 | 2 | 3 | 4 | 5 |
| 0.001 per cent thiamin (vitamin $B_1$) | A | 1 | 1 | 1 | 1 | 1 | 0.001 per cent nicotinic acid | A | 1 | 1 | 1 | 1 | 1 |
| | B | 1 | 1 | 1 | 2 | 2 | | B | 1 | 1 | 1 | 0 | 0 |
| | C | 1 | 1 | 1 | 1 | 1 | | C | 1 | 1 | 1 | 1 | 1 |
| | D | 2 | 2 | 1 | 1 | 1 | | D | 1 | 1 | 1 | 1 | 0 |
| | E | 1 | 1 | 1 | 0 | 0 | | E | 1 | 1 | 2 | 2 | 3* |
| 0.001 per cent riboflavin (vitamin $B_2$) | A | 1 | 1 | 1 | 1 | 1 | Mixture of equal parts of the solutions of thiamin, riboflavin, Factor 1, and nicotinic acid | A | 1 | 1 | 1 | 1 | 0 |
| | B | 1 | 1 | 1 | 1 | 1 | | B | 1 | 1 | 1 | 1 | 0 |
| | C | 1 | 1 | 1 | 1 | 1 | | C | 0 | 0 | 0 | 0 | 0 |
| | D | 1 | 2 | 2 | 2 | 2 | | D | 1 | 1 | 1 | 1 | 1 |
| | E | 1 | 1 | 1 | 0 | 0 | | E | 1 | 1 | 1 | 1 | 1 |
| 0.00025 per cent Factor 1 (vitamin $B_6$) | A | 1 | 1 | 1 | 1 | 0 | Standard yeast medium with thiamin destroyed | A | 2 | 16 | 62 | 150+ | 200+ |
| | B | 1 | 1 | 1 | 1 | 1 | | B | 2 | 33 | 90+ | 200+ | 200+ |
| | C | 2 | 2 | 2 | 2 | 1 | | C | 2 | 23 | 80+ | 200+ | 200+ |
| | D | 1 | 1 | 1 | 0 | 0 | | D | 2 | 2 | 0 | 0 | 0* |
| | E | 1 | 1 | 1 | 1 | 0 | | E | 3 | 24 | 80+ | 200+ | 200+ |

* Contaminated.

that feed on them. A possibility remained, however, that this source of thiamin might not be sufficient for the carnivores and that an additional supply might be required in the medium. This possibility was tested by autoclaving the standard medium at pH 9.6 for an hour to destroy all thiamin in the medium and then readjusting to pH 7.4 for use. Standard medium so treated supported excellent growth of both hypotrichs feeding on *Tetrahymena*. The destruction of thiamin in the standard medium did, however, have a slight effect indirectly on the maximum concentration of hypotrichs, owing to the limitation of the growth of the food ciliates. The fact that the effectiveness of standard medium is not directly affected by the destruction of thiamin may be seen in the results of the control cultures listed in Table 4.

In the case of the other vitamins, complete destruction or removal from the standard medium is not at present feasible, so that little has yet been learned of the actual re-

quirements for these vitamins either in the food ciliates or in the carnivores. It is possible that some or all of the vitamins of the B complex may be required in conjunction with the supplementary growth factor. There can be little doubt, however, that the supplementary growth factor is not identical with these vitamins. At least for the present it must be tentatively added to the list of chemically unidentified growth promoting substances found in yeast.

Some yeast preparations apparently do not contain the supplementary growth factor Bacto-Yeast Extract, a good source of most vitamins and growth factors, is apparently lacking in this particular factor. With the co-operation of the Difco Laboratories, manufacturers of the product, tests were made on various samples from different stages of its preparation. The samples taken from the first stages all showed growth-promoting activity for *Stylonychia* and *Pleurotricha;* but, at the point where a filter-aid (Supercel) was added, a considerable loss in activity was found. Samples taken after the removal of the filter mass did not support growth of the hypotrichs. From these preliminary experiments the removal of the growth factor appears to be an adsorption phenomenon, perhaps similar to adsorption on other materials discussed later. Taylor and van Wagtendonk (1941) have also found Bacto-Yeast Extract deficient when substituted for their brewers' yeast extract in media for the pure culture of *Colpoda*.

### THE NUTRITIONAL DEFICIENCY OF THE FOOD CILIATES

The foregoing experiments furnish abundant proof that a diet of *Tetrahymena* alone is inadequate for these carnivorous hypotrichs. In the case of another kind of inadequacy, involving another species of ciliate, *G. scintillans*, the organisms were never ingested. Hence observations were made on hanging-drop cultures to determine if ingestion of *Tetrahymena* occurred in the absence of the growth factor. The results of these experiments indicate that the absence of the supplementary growth factor does not immediately prevent ingestion of *Tetrahymena* by *Stylonychia*. The nutritional deficiency in this case appears to be quite different from that in the case of *G. scintillans*. In experiments testing another aspect of the nutritional deficiency of the food ciliates it was found that *Tetrahymena* previously grown on a medium containing the supplementary growth factor did not support growth of the carnivorous hypotrichs in a deficient medium. The supplementary growth factor, therefore, probably is not stored or concentrated in the food ciliates for appreciable periods of time but must be present in the medium itself for continued growth.

### ATTEMPTS AT PURIFICATION AND CONCENTRATION

In an attempt to concentrate and to purify the supplementary growth factor, Brewers' Yeast—Harris (medicinal), the richest and most convenient source, was extracted successively with ether, absolute alcohol, 95 per cent alcohol, 70 per cent alcohol, 50 per cent alcohol, and water. No growth of the carnivorous ciliates was obtained with the fractions from the ether, absolute alcohol, or 95 per cent alcohol extractions. The yield of dry material from these extractions was likewise very small. On the other hand, there was a considerable yield of dark-brown material from both the 70 per cent and 50 per cent alcohol extractions. Both fractions in the first tests proved to be quite similar in their growth-promoting effects, so that subsequently they were mixed together before the solvent was evaporated. The dry material was weighed and dissolved in distilled water to give a stock solution of the alcohol fraction of 0.1 per cent. Dilutions of this stock

solution were then tested for growth-promoting effects. The threshold concentration for the continued growth of both *S. pustulata* and *Pl. lanceolata* through three transplants was 0.05 per cent. In similar tests with the aqueous extract the threshold concentration was 0.02 per cent for *Stylonychia* and 0.01 per cent for *Pleurotricha*. The small difference in the growth-promoting activity of the alcoholic and aqueous preparations was considered insignificant and perhaps indicates that the supplementary growth factor is almost equally soluble in water and in 70 per cent alcohol. Hence, as a method of purifying and concentrating the effective substance (or substances) the extraction of yeast by alcohol and water did not offer much promise.

A number of vitamins and other growth factors have been separated from their natural sources by adsorption on charcoal or fullers' earth. These adsorbents were also found to be very effective in removing the supplementary growth factor from the standard medium both in the dilution used for cultures and the more concentrated stock solution. Treatment of one liter of the stock solution with as little as 10 grams of Norit (animal charcoal) was found sufficient to prevent continued growth of the hypotrichs in the medium. Treatment with fullers' earth gave similar results. The filtrates were tested by the routine method, using the H strain of *T. geleii* as food organisms. No toxic effects were observed in any of the filtrates used; both the hypotrichs and the food ciliates survived as long as in distilled water. When the Norit used in treating the yeast medium was washed in distilled water and tested by the routine method, both hypotrichs and food ciliates ingested the fine particles to some extent, but no appreciable growth was observed. Hence it appears that the hypotrichs required the supplementary growth factor dissolved in the medium and were not able to utilize it when adsorbed on particles of charcoal. Recently several active preparations have been obtained from the Norit used in such adsorptions by eluting with dilute aqueous ammonium hydroxide or with ammoniacal methyl alcohol. Further work is still in progress on the purification and concentration of the supplementary growth factor by means of such elutions.

## Discussion

These studies on bacteria-free carnivorous hypotrichs have brought out several previously unsuspected facts and have also helped to throw new light on some of the previous work with these species and closely related forms. It should, of course, be emphasized that the cultural conditions which have been maintained in this work were far from those found in nature. On the contrary, they represent an attempt to control more completely some of the variables previously present in experimental work with these and closely related species. One interesting question that might be raised is whether such cultures might be maintained indefinitely on the bacteria-free ciliate diet. Since single, washed individuals were used in establishing cultures, every strain constituted a pure line. The old controversy on the vitality of pure lines under controlled environmental conditions could here be revived. So far as actual experience is concerned, no strain has been maintained under these conditions for more than a year. The loss of a strain or a number of strains, however, under the conditions required for maintaining these cultures cannot, with any degree of assurance, be ascribed to intrinsic causes. In comparison with the pure culture of saprozoic ciliates the technical problems of the culture of carnivorous forms are far from solved to complete satisfaction, and the methods described here constitute merely a tentative working system capable of great improvement. Thus it appears impossible at present to answer questions on the vitality of these pure lines. In general, a decline in

vitality from the initial vigor of freshly isolated organisms was noticed in practically all lines, but this decline was not progressive. After preliminary fluctuations the daily division rate within the strain often remained quite constant for months. In those lines which were lost the final decline appeared quite suddenly, rather than as a cumulative effect of continually operative intrinsic causes. This suggests that the loss of these cultures was due to unknown environmental causes.

Of especial interest was the specific requirement of these carnivorous ciliates for particular kinds of living organisms. Thus *T. geleii* or *E. gracilis* supported growth, while *G. scintillans* or *Ch. paramecium* did not. As a larger number of carnivorous forms are studied, perhaps more light will be thrown on the entire question of protoplasmic specificity so well exemplified by the feeding reactions of the ciliate protozoa. The high degree of specificity shown by *Stylonychia* and *Pleurotricha* in their feeding reactions does not, however, prevent them from being, at the same time, quite omnivorous over the entire range of microörganisms. Thus, *Stylonychia* feeds on bacteria, yeast, and flagellates. *Pleurotricha*, while incapable of living on yeast alone, can be cultured on bacteria or flagellates. In view of this versatility in utilizing living organisms of widely different kinds, the remarkable specificity that is manifested within each group is almost paradoxical. If it may be assumed that the feeding reactions of ciliates are based fundamentally on the chemical constitution of the protoplasm of the food organism, these omnivorous hypotrichs may prove to be very valuable as test animals in comparative investigations on the biochemical relationships of microörganisms of many kinds. Sterilization techniques now present no technical difficulties for exhaustive studies of this kind, because the maintenance of cultures on the bacteria-free ciliate diet provides a convenient source of sterile hypotrichs in any desired number and in comparable physiological condition.

The apparent nutritional requirement for living protoplasm by *Stylonychia* and *Pleurotricha* is not unusual, since most species of ciliates have been found incapable of utilizing killed food organisms. The fact that the ciliate food organisms can be killed by physical agents which of themselves do not bring about denaturation of the proteins, however, has made it possible to disprove one of the explanations offered for the difference in nutritional value between living and dead organisms. For example, any chemical change which occurs when *Tetrahymena* is killed at 37° C. by sudden exposure cannot be due to alteration of the proteins as a direct result of this temperature, since the same temperature is not lethal when gradually attained. This makes it appear probable that the autolytic processes (perhaps due to enzyme action within the protoplasm) that are known to occur at death are responsible for the changes which affect the nutritive value of the material. Thus, any method of killing probably affects living protoplasm in a way that changes its nutritive value for these ciliates.

The most significant results of the study of these bacteria-free cultures of carnivorous ciliates have concerned the supplementary growth factor. In view of all the cultural work on ciliates that has been done, the fact that this requirement has not previously been discovered probably has been due to three principal reasons. First, some species of ciliates evidently do not require this particular growth factor. This is undoubtedly true of *T. geleii* and *T. vorax* and may also be true of other species of ciliates upon which tests have not yet been made. Second, certain species of bacteria, when present in cultures, may provide a source of this growth factor. Tests made with several strains of bacteria and the results of accidental bacterial contaminations in the course of this work,

however, indicate that not all bacteria furnish the growth factor. *Paramecium aurelia* was found by Phelps (1934) to require a substance found in lettuce infusion in addition to the single strain of bacteria provided as food. Whether the substance effective in this case is the same as the supplementary growth factor described above remains to be seen, but the fact that lettuce infusion was its source makes this an interesting possibility. Third, many of the media that have been used in the culture of ciliates have contained the supplementary growth factor. Such was undoubtedly the reason why Giese and Alden (1938) were able to grow bacteria-free *Stylonychia* on pure cultures of *Tetrahymena* in lettuce infusion. Hay infusion, another favorite medium in the culture of ciliates on mixed flora of bacteria, also may have provided a source of the growth factor in all the experiments, too numerous to mention, where this medium was used to maintain stock cultures.

A number of interesting speculations concerning the identity of the supplementary growth factor may be made from the consideration of its natural occurrence. The fact that effective concentrations were found in infusions of a wide variety of plant materials indicates a rather wide distribution in plants generally. The richest natural source, however, was found in yeast, the source of many important vitamins and other growth factors. Especially significant were the negative results obtained with a wide variety of media made from animal tissues. Of particular interest was the failure of liver extract to support growth of the hypotrichs, although *Tetrahymena* grew well in the medium. This would seem to eliminate from consideration all those substances whose richest natural sources include liver as well as yeast. The failure of media containing blood cells and blood serum to furnish the supplementary growth factor also narrows the search to substances which do not occur in appreciable quantities in mammalian blood. The effective substance (or substances), on the basis of its natural occurrence, must be found in especially high concentrations in yeast cells, in lower but significant quantities in many plant materials, and in very low and insignificant quantities in animal organs and tissues.

A possibility that has been considered in connection with the supplementary growth factor is that the growth-promoting effects may have been due indirectly to effects on the food ciliates. Thus, *Tetrahymena* surviving in distilled water or other deficient media might very properly be considered physiologically different from those actually growing and reproducing under conditions where metabolic activities were such as to favor an increased production of new protoplasm. It is true that the standard yeast medium was slightly nutritive for *Tetrahymena*, while many of the deficient media were not. On the other hand, the destruction of thiamin in standard medium did not affect its growth-promoting properties for *Stylonychia* and *Pleurotricha* but did limit the growth of *Tetrahymena*, so that for practical purposes the medium was nonnutritive. Furthermore, many media did not support the growth of the hypotrichs but were excellent for the culture of the food ciliates (proteose-peptone, Bacto-Yeast Extract, Bacto-Liver infusion). If it is objected that there was excessive growth in these media, the fact that growth was limited in some cases by diluting the media and in other cases by destroying thiamin should answer such an objection. Then, too, among the infusions from plant materials there were several media, including hay infusion, which did not support growth of the food ciliates but were effective for the hypotrichs. Altogether, therefore, there seems to be sufficient evidence that the growth-promoting effects of the supplementary growth factor for *Stylonychia* and *Pleurotricha* are independent of the growth of the food ciliates

in the medium. Of course, this growth factor may still function by virtue of an effect on the food ciliates other than growth. If this is the case, it may well prove difficult to investigate. It has been shown that *Tetrahymena* was not able to store or to concentrate the supplementary growth factor in its protoplasm for any appreciable length of time. Hence, if the effective substance is made available to the hypotrichs through the agency of the food ciliates, it must be a very transitory phenomenon.

There seems to be little likelihood that toxic effects are responsible for the failure of hypotrichs to grow in media that have been interpreted as deficient in the supplementary growth factor. Survival of ciliates for from 1 to 3 days in a given medium appears to be sufficient evidence that the medium is nontoxic. In most cases organisms surviving in deficient media resembled those surviving in distilled water under comparable conditions.

The solubility of the supplementary growth factor is one of its most characteristic properties. Boiling water easily extracted the effective substance from yeast cells in less than 15 minutes. In the simple extractions used in this work the effective substance was found soluble in water and in ethyl alcohol in concentrations up to 70 per cent. Extractions with ether, absolute alcohol, and 95 per cent alcohol, on the other hand, were without effect, showing that the effective substance is probably not lipoid in nature. Only characteristically water-soluble substances, therefore, have been considered in the search for the identity of the supplementary growth factor.

Another property which served to eliminate many other substances from consideration was its stability when autoclaved over an hour at pH values over 9.6. The primary purpose for this treatment was the destruction of thiamin; but other thermolabile growth factors, if present, must also have been destroyed. The growth factors reported under the names "vitamin $B_3$," "vitamin $B_4$," and "Factor W," if really distinct from the established vitamins of the B complex, would also have been destroyed. The three known thermostable vitamins of the B complex were, of course, shown, by the experiments summarized in Tables 3 and 4, to be unable to substitute for the supplementary growth factor. Biotin, pantothenic acid, and *i*-inositol were also shown to be without effect. From the evidence so far obtained it is possible to state only that the substance effective in promoting the growth of hypotrichs on a bacteria-free ciliate diet is probably not identical with any known vitamin. Whether it is identical with any other known growth-promoting substance so far described remains to be determined.

In the preliminary work on the purification of the supplementary growth factor by adsorption and elution the results have so far indicated that the growth-promoting effects are due, at least in part, to some substance (or substances) which is adsorbed on charcoal or fullers' earth. The failure of Bacto-Yeast Extract to support growth of these hypotrichs appears to be due to a similar removal of the growth factor by the filter-aid used at one point in its preparation. Whether this substance which is removed is completely effective alone or whether some co-factor is also involved has not yet been determined.

A requirement for inorganic salts on the part of the carnivorous ciliates does not appear to explain the growth-promoting effects of the standard yeast medium and other successful media. Media containing physiologically important inorganic salts, such as the balanced salt solution used by Barker and Taylor (1931), did not support growth. It appears probable that the inorganic requirements of the hypotrichs are adequately supplied in all cases either by the food ciliates or by the traces of salts present in the medium, regardless of its content of the supplementary growth factor.

## Summary

1. Two species of facultatively carnivorous ciliates—*S. pustulata* and *Pl. lanceolata*—have been rendered bacteria-free by a modification of the transfer washing method in which the containers were inclosed in Cellophane bags before autoclaving.

2. These ciliates were successfully cultured by supplying them with certain species of sterile ciliates in a suitable medium.

3. For continued growth of the carnivorous ciliates it was found necessary to supply in the medium, in addition to the food organisms, a supplementary growth factor.

4. The supplementary growth factor was found in largest quantities in yeast cells and in effective concentrations in infusions of a wide variety of plant materials but was not found in effective concentrations in media from animal organs or tissues.

5. The supplementary growth factor is soluble in water and in 70 per cent ethyl alcohol, is stable to heat and alkali, and is adsorbed on charcoal and on fullers' earth.

6. The supplementary growth factor is not identical with any of the known vitamins of the B complex.

7. Living ciliates of the species *T. geleii*, *T. vorax*, and *C. campylum* are adequate as food for these species of carnivorous ciliates when the supplementary growth factor is provided in the medium, but under the same conditions *G. scintillans is* inadequate.

8. Ciliates killed by exposure to temperatures of 37° C. or 0° C. or by high concentrations of sodium chloride are inadequate as food for these species.

9. The flagellates *E. gracilis* and *A. klebsii* are adequate as food for these species under certain cultural conditions, but under the same conditions *Ch. paramecium* is inadequate.

10. The algae *St. bacillaris* and *Chl.* sp. and a number of single strains of bacteria did not support continued growth of these species.

11. Living yeast cells in distilled water are adequate as food for *S. pustulata* but not for *Pl. lanceolata.*


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# THE EFFECTS OF LIGHT ALONE AND PHOTODYNAMIC ACTION ON THE RELATIVE VISCOSITY OF AMOEBA PROTOPLASM[1]

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THERE is virtually no evidence indicating the nature of the action of the longer waves of light on protoplasm. The small amount of information available on this subject is all the more noteworthy in view of the fact that protoplasm is almost continuously being exposed to the stimulating action of visible light. In their review of the literature on the action of radiation Heilbrunn and Mazia (1936) repeatedly point out that the living colloid is vastly different from any known inanimate colloid. Hence, information about the action of light on protoplasm is best obtained by direct study of protoplasm itself and not by inference from the known effects of light on proteins or other nonliving colloids.

Weber (1927), investigating the effects of direct sunlight on the cells of the plant *Ranunculus ficaria*, obtained results which indicate that the viscosity of the protoplasm is higher in the light than in the dark. However, as Weber himself noted, the increased viscosity might have been due to an indirect effect of the sunlight. Earle (1928) observed that, in general, when blood is illuminated the protoplasm of leucocytes is at first liquefied and then coagulated; but he was not certain that these changes were due to direct action of the light. On the basis of microscopic observations investigators have suggested that the cessation of protoplasmic streaming, observed when amoebae are suddenly exposed to intense light, is due to gelation of the cortical protoplasm, i.e., gelation of the plasmagel. There has, however, been no direct evidence in favor of this point of view.

It has been known since 1900 that various types of cells are markedly affected by the combined action of visible light and a sensitizing substance, i.e., by photodynamic action (see Raab, 1900; Blum, 1941; and Alsup, 1941). Yet, up to the present time, no experimental studies have been directly concerned with the effect of photodynamic action on the colloidal properties of protoplasm.

Since changes in the fluidity or viscosity of protoplasm cannot be detected with any great amount of certainty by microscopic observations, viscosity determinations are clearly necessary if one is to be certain of the effects of visible light and photodynamic action on the physical state of protoplasm.

Several reasonably accurate methods of measuring protoplasmic viscosity are available to students of protoplasm (see Heilbrunn, 1928). One of these, the centrifuge method, has been used extensively in determining the changes produced in the relative vis-

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cosity of cells by such stimulating agents as ultra-violet rays, X-rays, mechanical agitation, heat, electric current, and hydrostatic pressure. This method of studying protoplasmic viscosity can readily be applied to the two protozoan forms, *Amoeba proteus* and *A. dubia*, and has already given reliable data about the changes produced in the viscosity of their protoplasm by various stimulating agents (see Heilbrunn and Daugherty, 1931, 1932, 1933; and Angerer, 1936, 1937, 1940).

The essential properties of the protoplasm are the same in the two species; but centrifugal studies of *A. proteus* afford information about the viscosity of cortical protoplasm (plasmagel), whereas similar studies of *A. dubia* furnish data about the viscosity of the main mass of protoplasm (plasmasol). Both species respond to sudden illumination and photodynamic action by cessation or retardation of movement (see Mast, 1932; and Hyman and Howland, 1940); and, as was mentioned above, it has been suggested that these responses involve changes in the viscosity of the protoplasm (see Mast, 1932; Folger, 1925; and Alsup, 1939). The results that are presented in the following pages are primarily concerned with the effects of visible light alone and of photodynamic action on the relative viscosity of *Amoeba* protoplasm and have been obtained by centrifugal determinations.

## MATERIALS AND METHODS

*Amoeba proteus* used in the experiments were taken from subcultures of strains originally obtained from either the Carolina Biological Supply Company or Dr. J. A. Dawson. All original cultures of *A. dubia* were obtained from Dr. Dawson. Most of the amoebae used were cultivated in glass bowls containing 30–100 cc. of a modified Ringer's solution (see Hopkins, 1926) or spring water, to which either raw hay and wheat (one grain) or two grains of rice had been added. Most of the modified Ringer's solution used was made up with water distilled in a metal still; for some of the later experiemnts this solution was made up with water which had been redistilled in a fused silica still. In the ordinary distilled water, cultures of amoebae grew well, but the protoplasmic viscosity appeared to be high. A few experiments were performed on amoebae that had been raised in culture solutions made by adding raw hay and wheat to water from the metal still or to boiled tap water. Cultures in excellent condition were continuously at hand.

The apparatus used in subjecting the amoebae to illumination consisted essentially of a compound microscope and a 1,000-watt Mazda electric bulb mounted in an asbestos-lined wood ventilating shaft. The microscope rested on a shelf situated below a circular aperture in the front wall of the shaft, 50 mm. in diameter. The microscope, with the substage condenser removed, was so placed and the substage mirror so adjusted that the image of the glowing bulb filament was reflected up through the stage. The intensity of the light (the brightest portion) just above the stage was found to be approximately 300,000-meter candles when measured with a Weston photometer. The electric current used in the bulb was drawn from a 115-volt alternating-current lighting circuit. During the experiments the temperature around the front wall of the shaft varied very little from that of the room in which the experiments were carried out. This constancy of temperature was obtained by allowing the light rays to pass through distilled water for absorption of heat and in most of the experiments by the additional cooling action of an electric fan situated in the ventilating shaft.

The relative viscosity of the plasmagel of irradiated and nonirradiated *A. proteus* was determined with an Emerson hand centrifuge. This instrument developed a centrifugal

force 2,474 times gravity when the handle was turned at the rate of one revolution per 2 seconds. Determinations of the relative viscosity of the plasmasol of *A. dubia* were made with an old-model Bausch and Lomb hand centrifuge of the same type as that employed by Heilbrunn and Daugherty (1931, 1933). A centrifugal force 512 times gravity was sufficient for determining the relative viscosity of the rather fluid plasmasol of *A. dubia*.

The centrifugal values that are recorded in this paper for *A. proteus* represent the smallest number of complete revolutions that the handle of the Emerson centrifuge had to be turned in order to shift the crystals of the plasmagel into one-half of the cell. The centrifuge values for *A. dubia* represent the shortest time in seconds required for the crystals of the plasmasol to move through one-half of the diameter of the cell under a centrifugal force 512 times gravity. The centrifuge values recorded for any given experiment were obtained only after a series of determinations. Definitive centrifuge values were not considered reached unless the crystals in at least 80 per cent of the specimens examined showed the specified degree of movement. Because of the differences in relative viscosity of amoebae from different cultures and because of the remarkable constancy of viscosity of amoebae from the same culture, the amoebae used in any given experiment were always taken from the same culture.

In those experiments in which amoebae were immersed in pure salt solutions before being irradiated and centrifuged, all speciments were washed at least once in the solutions. Those solutions whose effects on the action of light were to be tested were made up with Merck's blue-label salts and water, redistilled in the fused silica still.

Only two photodynamically active dyes, rose bengal and eosin, were used in the experiments designed to elucidate the effect of photodynamic action on the viscosity of the plasmagel and plasmasol. Both dyes were obtained from the Coleman and Bell Company. Stock solutions of each dye were made up in the proportions of 1 part dye to 2,000 parts modified Ringer's solution. In the experiments with eosin, only the stock solution was used. More dilute solutions of rose bengal were obtained by adding modified Ringer's solution to the stock solution.

In the course of each photodynamic experiment amoebae were transferred from the culture dish to a small Stender dish. Most of the culture fluid carried over in the transfer was removed by means of capillary pipettes, and then 10–25 cc. of the dye solutions were added in the dark to the dish. Amoebae were left in the dark in the various dye solutions for 1–6 hours before they were irradiated or centrifuged.

The amoebae to be studied were taken from the culture fluids or the experimental solutions and placed in glass centrifuge tubes by means of capillary pipettes. Those amoebae that were not to be irradiated were usually centrifuged immediately after being placed in the tubes. Pairs of tubes containing amoebae to be subjected to illumination were laid on the stage of the microscope in such a way that the organisms were directly over the brightest portion of the image of the bulb filament. In some experiments the pairs of tubes were held before the aperture in the front wall of the ventilating shaft so that they were directly in front of the glowing filament. Essentially the same results were obtained with these two methods of exposing amoebae to the light. The exposure time for most experiments was 2 minutes. The irradiated amoebae were centrifuged at various times after the exposures, depending on the type of experiment being done. All centrifuged amoebae were examined under a second microscope as fast as they could be removed from the centrifuge tubes to a glass slide on the stage of this microscope. In most

experiments the interval of time between the end of the centrifugation period and the beginning of the observation period did not exceed 10 seconds.

## EXPERIMENTAL RESULTS

### I. THE EFFECT OF LIGHT ALONE ON *Amoeba* PROTOPLASM

*a) Changes in the viscosity of the plasmagel of A. proteus immersed in culture fluids and pure salt solutions.*—When specimens of *A. proteus* immersed in culture fluids are exposed to intense visible radiation, the relative viscosity of the plasmagel is markedly increased. This is clearly shown by the data in Table 1. In this table each line is the summarized result of a series of tests on the effect of light on a given culture of amoebae. Normal centrifuge values for amoebae from various cultures generally fell be-

TABLE 1

THE ACTION OF LIGHT ON THE RELATIVE VISCOSITY OF THE PLASMAGEL OF *A. proteus* TESTED IMMEDIATELY AFTER IRRADIATION

(Time exposed to light, 2 minutes; temperature range, 19°–23° C.)

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Percentage Increase in Viscosity after Exposure to Light | Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Percentage Increase in Viscosity after Exposure to Light |
|---|---|---|---|---|---|---|---|
| 1 | 90 | 135 | 50 | 10 | 125 | 155 | 24 |
| 2 | 120 | 150 | 25 | 11* | 120 | 190 | 58.3 |
| 3 | 65 | 90 | 38 | 12* | 95 | 130 | 35 |
| 4 | 95 | 125 | 31.5 | 13 | 100 | 135 | 35 |
| 5 | 105 | 125 | 19 | 14 | 95 | 140 | 48 |
| 6 | 150 | 175 | 16.6 | 15 | 125 | 165 | 32 |
| 7 | 120 | 160 | 33 | 16 | 105 | 135 | 28.5 |
| 8 | 125 | 165 | 32 | 17 | 90 | 135 | 50 |
| 9 | 150 | 190 | 26.6 | | | | |

* Time exposed to light, 1 minute.

tween 90 and 125. The centrifuge values of the irradiated amoebae are seen to be from 16.6 to 58.3 per cent greater than those for nonirradiated amoebae, with most of the values being about 30 per cent greater. The centrifuge tests on all irradiated amoebae considered in Table 1 were begun 7–10 seconds after the exposures, i.e., as fast as it was possible to place the tubes in the arms of the centrifuge.

Examination of the data in Table 2 shows that the increase in viscosity of the plasmagel, which can be detected in 7–10 seconds after the exposure of the amoebae to light, persists for a considerable length of time. The normal centrifuge values for amoebae from various cultures are given in the second column of this table. The third, fourth, fifth, sixth, seventh, and eighth columns contain the centrifuge values for amoebae from these cultures at various times after their irradiation. Upon comparing the values in the second column with those in the last six columns, it becomes evident that in all cases the viscosity did not return to the normal level until about 30 minutes after the exposures to light. Moreover, the viscosity values, 1, 5, and 15 minutes after irradiation, are seen to be approximately the same as those at the end of the regular 7–10-second period.

The experimental results given in Tables 1 and 2 were obtained with amoebae immersed in culture fluids which contained various inorganic and organic compounds. Heilbrunn and Daugherty (1932) have shown that each of the four common cations—namely, sodium, potassium, magnesium, and calcium—has a definite effect on the viscosity of the plasmagel of *A. proteus*. The chlorides of sodium, potassium, and magnesium decrease the viscosity of the plasmagel, whereas calcium chloride increases it. Since visible light alters the colloidal state of the plasmagel in the same direction that calcium does, quite logically the question arises as to whether calcium ions are involved in the gelating action of light. The answer to this question can be ascertained experimentally by determining the effect of light on the plasmagel of amoebae immersed either in a solution of a calcium-binding or of a calcium-removing substance, like ammonium oxalate, or in

TABLE 2

THE VISCOSITY OF THE PLASMAGEL OF *A. proteus* AT VARIOUS TIMES AFTER EXPOSURE TO LIGHT

(Time exposed to light, 2 minutes; temperature range, 18°–24° C.)

| Expt. No. | Normal Centrifuge Value | Centrifuge Values after Exposures to Light | | | | | |
|---|---|---|---|---|---|---|---|
| | | 7–10 Seconds after Exposure | 1 Minute after Exposure | 5 Minutes after Exposure | 15 Minutes after Exposure | 30 Minutes after Exposure | 45 Minutes after Exposure |
| 1 | 105 | 135 | 145 | 148 | 148 | 108 | 105 |
| 2 | 110 | 165 | 168 | 165 | 168 | 115 | 105 |
| 3 | 90 | 138 | 145 | 140 | 135 | 95 | 100 |
| 4 | 60 | 85 | 85 | 90 | 85 | 70 | 70 |
| 5 | 110 | 140 | 145 | 140 | 145 | 120 | 118 |
| 6 | 115 | 160 | 165 | 165 | 165 | 135 | 120 |
| 7 | 95 | 120 | 125 | 128 | 120 | 100 | 95 |
| 8 | 95 | 115 | 115 | 115 | 120 | 95 | 95 |
| 9 | 55 | 70 | 70 | 75 | 68 | 55 | 55 |
| 10 | 120 | 155 | 155 | 160 | 155 | 120 | 130 |

solutions containing calcium-replacing ions, like sodium or potassium. In addition, experiments performed with amoebae immersed in pure solutions of sodium or potassium chloride should reveal whether these two cations bear any essential relationship to the effect of light on viscosity. Experiments designed to obtain answers to these questions were made, and the results obtained are presented in Tables, 3, 4, and 5.

Table 3 shows the effect of light on the viscosity of the plasmagel of amoebae immersed in a M/40 solution of potassium chloride. The fifth column of this table contains the centrifuge values for amoebae immersed in the solution but not irradiated, whereas the seventh column lists the centrifuge values for amoebae irradiated for 2 minutes after immersion in the potassium chloride solution for the lengths of time given in the sixth column. Upon comparing the values in the fourth and fifth columns with the normal centrifuge values given in the second column, it may be seen that the plasmagel is much more fluid after long immersions (45–90 minutes) in potassium chloride solution than after short immersions (15–45 minutes) in this solution. A comparison of the values in the fourth and fifth columns with those in the sixth and seventh columns, respectively,

reveals that light can increase the viscosity of the plasmagel after it has been lowered by short immersion in potassium chloride solution but not after it has been lowered further by long immersion in this solution.

As the results presented in Table 4 show, sodium bears essentially the same relationship to the action of light that potassium does. The viscosity of the plasmagel can be in-

TABLE 3

THE EFFECT OF LIGHT ON THE VISCOSITY OF THE PLASMAGEL OF *A. proteus* IMMERSED IN M/40 POTASSIUM CHLORIDE SOLUTION

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Time in KCl in Minutes | Centrifuge Value after Exposure to KCl | Time in KCl in Minutes | Centrifuge Value after Exposure to Light and KCl |
|---|---|---|---|---|---|---|
| 1 | ..... | ..... | 15–25 | 50 | 15–25 | 75 |
| 2 | ..... | ..... | 15–25 | 75 | 15–25 | 110 |
| 3 | ..... | ..... | 15–25 | 70 | 15–25 | 120 |
| 4 | ..... | ..... | 15–25 | 70 | 15–25 | 90 |
| 5 | ..... | ..... | 15–25 | 100 | 15–25 | 120 |
| 6 | 125 | 150 | 45 | 100 | 45 | 140 |
| | | | 120 | 50 | 125 | 50 |
| 7 | 150 | 170 | 15 | 135 | 20 | 140 |
| | | | 60 | 60 | 60 | 60 |
| 8 | 125 | 170 | 20 | 105 | 20 | 125 |
| | | | 90 | 35 | 95 | 35 |
| 9 | 105 | 135 | 20 | 70 | 20 | 90 |
| | | | 60 | 45 | 60 | 45 |
| 10 | 125 | 160 | 20 | 100 | 30 | 135 |
| | | | 45 | 45 | 45 | 55 |
| 11 | 140 | 210 | 15 | 110 | 15 | 165 |
| | | | 60 | 80 | 75 | 75 |
| 12 | 90 | 120 | 25 | 75 | 30 | 90 |
| | | | 90 | 65 | 90 | 60 |
| 13 | 75 | 95 | 15 | 55 | 15 | 65 |
| | | | 55 | 20 | 65 | 25 |
| 14 | 75 | 95 | 18 | 45 | 25 | 60 |
| | | | 60 | 30 | 60 | 33 |
| 15 | 132.5 | 165 | 25 | 120 | 25 | 120 |
| | | | 90 | 40 | 90 | 40 |

creased by irradiation following short immersion of the amoebae in M/40 sodium chloride but not by irradiation following long immersion in this concentration of sodium chloride. It is interesting to note that sodium is not so effective as potassium either in lowering the viscosity of the plasmagel or in preventing the gelating action of light. The gelating action of light is prevented only after the amoebae have been immersed in the sodium chloride solution for very long periods, usually 2–3 hours; and even after these long immersions the decrease in viscosity due to the sodium is often slight.

Heilbrunn and Daugherty (1933) found that if calcium is removed from the plasmagel by immersing amoebae in M/32 ammonium oxalate solution the viscosity of this protoplasmic region tends to be lowered very markedly. The results given in Table 5 of the

TABLE 4

EFFECT OF LIGHT ON THE VISCOSITY OF THE PLASMAGEL OF *A. proteus* IMMERSED IN M/40 SODIUM CHLORIDE SOLUTION

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Time in NaCl in Minutes | Centrifuge Value after Exposure to NaCl | Time in NaCl in Minutes | Centrifuge Value after Exposure to NaCl and Light |
|---|---|---|---|---|---|---|
| 1 | 45 | 65 | 30 | 37.5 | 45 | 45 |
| | | | 110 | 27.5 | 115 | 30 |
| 2 | 145 | 180 | 45 | 135 | 50 | 160 |
| | | | 75 | 127.5 | 80 | 120 |
| 3 | 125 | 162.5 | 50 | 125 | 60 | 150 |
| | | | 190 | 85 | 200 | 90 |
| 4 | 132.5 | 170 | 50 | 125 | 60 | 150 |
| | | | 110 | 95 | 118 | 100 |
| 5 | 140 | 175 | 35 | 120 | 43 | 140 |
| | | | 108 | 120 | 115 | 125 |
| 6 | 120 | 155 | 25 | 100 | 32 | 135 |
| | | | 130 | 95 | 120 | 90 |
| 7 | 70 | 90 | 40 | 75 | 35 | 95 |
| | | | 125 | 55 | 130 | 50 |
| 8 | 145 | 175 | 20 | 140 | 60 | 145 |
| | | | 150 | 110 | 160 | 115 |
| 9 | 125 | 155 | 35 | 120 | 45 | 150 |
| | | | 190 | 110 | 176 | 95 |
| 10 | 110 | 145 | 50 | 90 | 60 | 105 |
| | | | 120 | 85 | 110 | 90 |

TABLE 5

THE EFFECT OF LIGHT ON THE VISCOSITY OF THE PLASMAGEL OF *A. proteus* IMMERSED IN M/32 AMMONIUM OXALATE SOLUTION

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Time in $NH_4$ Oxalate in Minutes | Centrifuge Value after Immersion in $NH_4$ Oxalate | Time in $NH_4$ Oxalate in Minutes | Centrifuge Value after Exposure to $NH_4$ Oxalate and Light |
|---|---|---|---|---|---|---|
| 1 | 125 | 155 | 60 | 80 | 75 | 80 |
| 2 | 80 | 110 | 60 | 25 | 65 | 27 |
| 3 | 80 | 110 | 46 | 30 | 46 | 30 |
| 4 | 70 | 95 | 60 | 25 | 70 | 20 |
| 5 | 90 | 120 | 45 | 55 | 45 | 65 |
| 6 | 110 | 135 | 50 | 40 | 50 | 45 |
| 7 | 105 | 135 | 76 | 55 | 70 | 60 |
| 8 | 75 | 110 | 30 | 35 | 80 | 30 |
| 9 | 105 | 135 | 72 | 30 | 80 | 30 |
| 10 | 80 | 110 | 50 | 25 | 55 | 23 |
| 11 | 80 | 125 | 6 | 35 | 8 | 35 |
| 12 | 135 | 175 | 8 | 45 | 12 | 40 |

present paper are in accord with this finding. Moreover, the data recorded in this table show quite clearly that visible light cannot gelate the plasmagel of amoebae immersed in oxalate solution, even though the immersion periods be relatively short.

*b) Changes in the viscosity of the plasmasol of A. dubia immersed in culture fluids and pure salt solution.*—Up to this point we have been concerned with the effect of light on the viscosity of the plasmagel of *A. proteus*. As mentioned above, the study of protoplasmic viscosity in amoebae has a particular advantage in that it is possible to determine not only the viscosity of the cortex or plasmagel but also that of the interior protoplasm or plasmasol. It should, therefore, be possible to discover whether exposure to intense light has any effect on the relative viscosity of the plasmasol of *A. dubia*. Such was, indeed, found to be the case, as is shown by the data in Table 6.

TABLE 6

THE EFFECT OF LIGHT ON THE RELATIVE VISCOSITY OF THE PLASMASOL OF *A. dubia*

(Temperature, 20°–25° C.)

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Exposure Time in Minutes | Percentage Increase in Viscosity after Exposure to Light |
|---|---|---|---|---|
| 1 | 9 | 22 | 2 | 140 |
| 2 | 7 | 5 | 2 | − 28 |
| 3 | 4 | 11 | 2 | 175 |
| 4 | 10 | 23 | 2 | 130 |
| 5 | 4 | 7 | 2 | 75 |
| 6 | 2 | 4 | 2 | 100 |
| 7 | 9 | 21 | 2 | 132 |
| 8 | 7 | 17 | 2 | 143 |
| 9 | 5 | 11 | 3 | 120 |
| 10 | 13 | 25 | 2 | 92 |
| 11 | 6 | 11 | 3 | 83 |
| 12 | 5 | 15 | 4 | 200 |
| 13 | 10 | 21 | 4 | 110 |
| 14 | 5 | 16 | 2 | 220 |
| 15 | 6 | 6 | 3 | 0 |

Comparison of the values in the second and third columns of this table shows that visible radiation has a real gelating effect on the plasmasol. After irradiation the centrifuge values of the plasmasol of the amoebae considered in this table showed an average increase of 123 per cent over the normal values when intervals of 7–10 seconds elapsed between cessation of irradiation and the beginning of the centrifuge tests. In a few (five) experiments determinations of the centrifuge values were begun 30, 120, and 180 seconds after the cessation of irradiation. The increases in centrifuge values detected after these longer intervals were of the same order of magnitude as those noted after the regular 7–10-second intervals. In other words, the centrifuge values remained constant at the increased values at least until an interval of 180 seconds had elapsed between cessation of irradiation and beginning of centrifugation.

In the case of the plasmagel, it has been seen that calcium which itself gelates the

plasmagel is essential for the gelating action of light on the plasmagel. Calcium tends to make the plasmasol less viscous, although in excess it presumably makes it more viscous (Heilbrunn and Daugherty, 1933). This being true, it seemed worth while to find out what effect removal of cacium from the plasmasol has on the gelating action of light. However, before considering experimental data bearing on this question, it should be mentioned that the removal of calcium from the interior of an amoeba is probably a more complex operation than the removal of calcium from the cortex of an amoeba. It seems reasonable to suppose that oxalate produces few, if any, secondary effects when it removes calcium from the outer portion of a cell; but this is most likely not the case when

TABLE 7

THE EFFECT OF LIGHT ON THE VISCOSITY OF THE PLASMASOL OF *A. dubia* IMMERSED IN M/32 AND M/40 AMMONIUM OXALATE SOLUTIONS

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light | Time in $NH_4$ Oxalate in Minutes | Centrifuge Value after Exposure to $NH_4$ Oxalate | Time in Oxalate in Minutes | Centrifuge Value after Exposure to $NH_4$ Oxalate and Light | Concentration of $NH_4$ Oxalate |
|---|---|---|---|---|---|---|---|
| 1 | 7 | 12 | 50 | 3 | 45 | 3 | M/32 |
| 2 | 10 | 20 | 60 | 8 | 60 | 8 | M/32 |
| 3 | 2 | 4 | 75 | 4 | 60 | 4 | M/32 |
| 4 | 15 | 31 | 20 | 20 | 25 | 20 | M/32 |
| 5 | 17 | 22 | 55 | 26 | 65 | 20 | M/32 |
| 6 | 8 | 16 | 15 | 4 | 22 | 3 | M/32 |
| | | | 100 | 4 | 103 | 3 | M/32 |
| 7 | 8 | 15 | 50 | 11 | 60 | 8 | M/32 |
| 8 | 4 | 8 | 15 | 10 | 20 | 9 | M/32 |
| 9 | 5 | 8 | 45 | 4 | 55 | 4 | M/32 |
| | | | 130 | 4 | 125 | 4 | M/32 |
| 10 | 9 | 16 | 70 | 11 | 75 | 11 | M/40 |
| 11 | 7 | 13 | 45 | 6 | 55 | 5 | M/40 |
| | | | 105 | 6 | 100 | 5 | M/40 |
| 12 | 10 | 18 | 60 | 4 | 67 | 4 | M/40 |
| 13 | 12 | 19 | 80 | 13 | 90 | 12 | M/40 |
| 14 | 10 | 20 | 75 | 5 | 65 | 5 | M/40 |

oxalate must remove calcium from the cell interior. A good indication that the removal of calcium from the interior of an amoeba is not a simple process comes from the experimental finding that ammonium oxalate has a variable effect on the viscosity of the plasmasol of *A. dubia*. This is seen when the values in the second column of Table 7 are compared with those in the fifth column of the same table. In some experiments the oxalate lowered, in others increased, and in still others had little effect on the viscosity of the plasmasol. Since the amoebae used in the experiments reported in Table 7 had been raised in culture fluids which varied considerably in electrolyte content, it seemed possible that the manner in which ammonium oxalate affects the viscosity of the plasmasol is, to a large extent, determined by the electrolytes present in the original environment of the amoebae. Indeed, some preliminary experiments indicate that this is true and that calcium and magnesium ions in the culture fluids are the ions of primary importance in determining just how oxalate will affect the viscosity of the plasmasol.

Even though it is not clear just what secondary effects oxalate may have on the interior of an amoeba, there can be little doubt that oxalates prevent the free movement of calcium in a cell. Therefore, if oxalate solutions have a consistent effect on the gelation of the plasmasol by visible light, one can safely attribute this effect to the binding or removal of free calcium. Ammonium oxalate does have a consistent effect on the gelating action of light on the plasmasol of *A. dubia*. It prevents the action of light. This may be seen from the data in Table 7. In this table the centrifuge values of the irradiated oxalated amoebae must be compared not with the centrifuge values of the normal amoebae but with the centrifuge values of the control oxalated amoebae which were not subjected to radiation. These values are given in the fifth and seventh columns. A comparison shows that irradiation produces no appreciable increase in the viscosity of oxalated amoebae. Thus the gelation of plasmasol by light, as well as the gelation of the plasmagel by light, is dependent upon the presence of free calcium in the amoebae.

### II. THE EFFECT OF PHOTODYNAMIC ACTION ON *Amoeba* PROTOPLASM

As mentioned above, fluorescent dyes, like rose bengal and eosin, make various types of cells more sensitive to visible light. Amoebae immersed in weak solutions of rose bengal or eosin are readily cytolyzed by short exposure to intense light, whereas amoebae immersed in culture solutions are not killed by prolonged exposures to light of the same intensity. In the dark, weak solutions of rose bengal and eosin are relatively nontoxic to amoebae. This is evident inasmuch as amoebae live as long as a week in such solutions. Thus, when acting alone, intense light and weak dye solutions have little or no injurious effects on amoebae; but when acting together, i.e., photodynamically, these two agents very soon cause the death of the amoebae.

The effects of weak solutions of the two photodynamically active dyes and photodynamic action on the protoplasm of *A. proteus* and *A. dubia* have been studied, and the results obtained are considered in the two following sections of this paper.

*a) Changes in the viscosity of the plasmagel of A. proteus immersed in weak solutions of rose bengal and eosin.*—Table 8 summarizes the results of a series of tests on the effects of photodynamically active dyes and photodynamic action on the relative viscosity of the plasmagel of *A. proteus*. In this table the second and third columns, respectively, give the centrifuge values for nonirradiated and irradiated control amoebae immersed in culture fluids. The centrifuge values of amoebae immersed in dye solutions but not irradiated are given in the sixth column. The last column of the table gives the centrifuge values of amoebae exposed for 2–5 minutes to light from the 1,000-watt bulb after having been immersed in the dye solutions for the time given in the seventh column. It can be seen at a glance that in every case exposure to photodynamic action causes a drop in the centrifuge value of the plasmagel. On the average, the centrifuge value of the plasmagel of amoebae exposed to light while immersed in dye solutions is 23 per cent lower than that of normal amoebae. Comparison of the values in the sixth column with those in the second column brings out the interesting fact that amoebae immersed for 2–6 hours in the dark in 1:10,000 and 1:50,000 rose bengal and in 1:2,000 eosin have practically the same centrifuge values as normal amoebae. In other words, the dyes alone have little or no effect on the viscosity of the plasmagel. Thus, when acting separately, light and the dyes respectively increase and leave unaltered the viscosity of the plasmagel of *A. proteus* but, when acting together, cause a real liquefaction of the plasmagel.

This liquefaction of the plasmagel persists for quite some time after the exposures to photodynamic action. In all of the experiments listed in Table 8 the centrifuge values were determined 7–10 seconds after the exposures. In nine of these experiments a second set of centrifuge values was determined 4–15 minutes after the exposures. In each of these nine cases the centrifuge values were approximately the same after the longer intervals as after the regular 7–10-second intervals. It is interesting to note that normal protoplasmic flow usually began immediately after the short exposures were over.

*b*) *Changes in the viscosity of the plasmasol of A. dubia immersed in weak solutions of rose bengal and eosin.*—Table 9 presents the results obtained from fourteen experiments designed to ascertain what effect the two dyes, rose bengal and eosin, have on the viscos-

TABLE 8

THE EFFECT OF PHOTODYNAMIC ACTION ON THE VISCOSITY OF THE PLASMAGEL OF *A. proteus*

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light Alone | Concentration of Dye* | Time in Dye in Minutes | Centrifuge Value after Exposure to Dye Alone | Time in Dye in Minutes | Centrifuge Value after Exposure to Dye and Light |
|---|---|---|---|---|---|---|---|
| 1 | 85 | 110 | 1:50,000 | 140 | 85 | 130 | 60 |
| 2 | 120 | 150 | 1:50,000 | 175 | 112.5 | 165 | 80 |
| 3 | 50 | 65 | 1:50,000 | 105 | 57.5 | 120 | 37.5 |
| 4 | 145 | 175 | 1:50,000 | 190 | 142.5 | 200 | 105 |
| 5 | 160 | 190 | 1:50,000 | 165 | 160 | 175 | 115 |
| 6 | 45 | 60 | 1:50,000 | 90 | 42.5 | 120 | 32.5 |
| 7 | 122.5 | 165 | 1:50,000 | 135 | 135 | 140 | 90 |
| 8 | 80 | 105 | 1:50,000 | 180 | 85 | 200 | 67.5 |
| 9 | 65 | 95 | 1:50,000 | 110 | 55 | 120 | 35 |
| 10 | 175 | 230 | 1:10,000 | 110 | 175 | 115 | 105 |
| 11 | 300 | 410 | 1:10,000 | 100 | 320 | 180 | 240 |
| 12 | 80 | 95 | 1:10,000 | 180 | 75 | 175 | 50 |
| 13 | 95 | 120 | 1:10,000 | 300 | 120 | 305 | 75 |
| | | | 1:2,000 E† | 340 | 100 | 355 | 75 |
| 14 | 165 | 205 | 1:10,000 | 305 | 170 | 315 | 115 |
| | | | 1:2,000 E† | 285 | 165 | 275 | 120 |

* Concentration of rose bengal expressed as ratio of parts of dye to parts of modified Ringer's solution.
† Concentration of eosin expressed as ratio of parts of dye to parts of modified Ringer's solution.

ity of the plasmasol of *A. dubia* when acting alone and in combination with light from the 1,000-watt bulb. The procedure for these experiments was identical with that for the experiments just described, except that the smaller hand centrifuge was used to measure the relative viscosity of the rather fluid plasmasol. The data in Table 9 are arranged in exactly the same manner as those in Table 8. Examination of Table 9 shows that the results obtained with *A. dubia* are quite similar to those obtained with *A. proteus*. The average centrifuge value of the plasmasol of amoebae immersed for 1–5 hours in the dark in 1:10,000 and 1:50,000 rose bengal and 1:2,000 eosin is approximately the same as that of normal amoebae. The centrifuge values of the plasmasol of amoebae exposed to light for 0.5–2.0 minutes while immersed in the dye solutions are seen to be lower than those of normal amoebae. On the average, the centrifuge value of the plasmasol of amoebae exposed to photodynamic action is only a little over a half that of normal

amoeba. It should be noted that photodynamic action lowers the viscosity of the plasmasol of *A. dubia* much more sharply than it does that of the plasmagel of *A. proteus*. Moreover, just as was true for the plasmagel, the drop in viscosity of the plasmasol, which can be detected in 7–10 seconds after the exposure to photodynamic action, persists for considerable lengths of time (4–28 minutes) after the exposure. Protoplasmic flow usually began soon after the exposure was over.

TABLE 9

THE EFFECT OF PHOTODYNAMIC ACTION ON THE RELATIVE VISCOSITY OF THE PLASMASOL OF *A. dubia*

| Expt. No. | Normal Centrifuge Value | Centrifuge Value after Exposure to Light Alone | Concentration of Dye* | Time in Dye in Minutes | Centrifuge Value after Exposure to Dye Alone | Time in Dye in Minutes | Centrifuge Value after Exposure to Dye and Light |
|---|---|---|---|---|---|---|---|
| 1 | 5 | 10 | 1:50,000 | 180 | 6 | 180 | 2.5 |
| 2 | 13 | ..... | 1:50,000 | 200 | 12 | 210 | 9 |
| 3 | 10 | 14 | 1:50,000 | 240 | 10 | 260 | 7 |
| 4 | 2 | 5 | 1:50,000 | 280 | 2 | 300 | 1 |
| 5 | 11 | 16 | 1:50,000 | 160 | 9 | 180 | 5 |
| 6 | 9 | 14 | 1:50,000 | 150 | 9 | 130 | 5 |
| 7 | 11 | 16 | 1:50,000 | 200 | 12 | 200 | 5 |
| 8 | 12 | 16 | 1:50,000 | 120 | 12 | 130 | 6 |
| 9 | 12 | 17 | 1:50,000 | 150 | 12 | 170 | 5 |
| 10 | 9 | 16 | 1:50,000 | 120 | 9 | 180 | 7 |
| 11 | 11 | 16 | 1:50,000 | 210 | 11 | 220 | 7 |
| | | | 1:10,000 | 194 | 11 | 190 | 5 |
| | | | 1:2,000 E† | 132 | 11 | 198 | 6 |
| 12 | 10 | 10 | 1:50,000 | 250 | 10 | 260 | 5 |
| 13 | 6 | 8 | 1:50,000 | 220 | 6 | 280 | 3 |
| | | | 1:10,000 | 250 | 6 | 290 | 2 |
| | | | 1:2,000 E† | 305 | 6 | 270 | 3 |
| 14 | 4 | 8 | 1:10,000 | 100 | 4 | 120 | 2 |
| | | | 1:2,000 E† | 100 | 5 | 180 | 2 |

* Concentration of rose bengal expressed as ratio of parts of dye to parts of modified Ringer's solution.

† Concentration of eosin expressed as ratio of parts of dye to parts of modified Ringer's solution.

## DISCUSSION

It is interesting to note that, whereas light and photodynamic action have consistent effects on both the plasmagel and the plasmasol of amoebae—light causing gelation and photodynamic action a liquefaction of each of these regions—this is not true for stimulating agents like ultra-violet light, mechanical agitation, and electric current. The latter three stimulating agents have been found to solate, as well as gelate, the plasmasol of *A. dubia* and to cause only a liquefaction of the plasmagel of *A. proteus* (see Heilbrunn and Daugherty, 1933; and Angerer, 1936 and 1937). However, visible light and ultra-violet do have one property in common: neither of these two stimulating agents can alter the viscosity of the plasmagel or plasmasol of oxalated amoebae. In view of the fact that oxalates remove calcium from protoplasm, the conclusion has been drawn that calcium must be essential for the actions of visible and ultra-violet light on amoeba protoplasm. Although no study has been made of the effect of photodynamic action on

the viscosity of the protoplasm of oxalated amoebae, some experimental results obtained with the eggs of *Nereis limbata* indicate that photodynamic effects are also conditioned by the removal of calcium from cells (see Alsup, 1941). Doubtless, factors other than calcium are involved in the liquefying and gelating effects of photodynamic action and visible light, respectively; but my results indicate that any explanation of the stimulating action of these two agents must take into consideration the role of calcium. Therefore, it appears worth while to discuss the experimental results presented in this paper from the standpoint of Heilbrunn's calcium-release theory of general stimulation (see Heilbrunn, 1937).

This theory holds that ordinary stimulating agents cause the breakdown of a cortical calcium-containing gel, with the resultant passage to the cell interior of free calcium ions. It is assumed that this release of calcium from the cortical protoplasm or plasmagel occasions the liquefaction of this layer and that the released calcium upon entering the interior of the cell causes first a liquefaction and then a gelation of the inner protoplasm (plasmasol). My results indicate that calcium is involved in the action of visible light on amoeba protoplasm, but they show clearly that light does not produce the shift of calcium ions which results in such a series of viscosity changes in amoeba as is outlined by the calcium-release theory. Intense visible radiation causes a gelation of both the cortical and the interior protoplasm of the amoeba. Now, if calcium is responsible for the stiffening of the plasmagel of the amoeba, this would seem to indicate that light is able to release calcium from some combination in the interior of the amoeba. In other words, it appears that in the amoeba there is a source of calcium other than the one in the cortex, a source that light may be able to affect because of its ability to penetrate the protoplasm of the amoeba. If there is a source of calcium in the interior of the amoeba, light might be expected to release calcium from this source and thereby effect a gelation of the plasmagel even when the calcium content of this outer region has been reduced by immersion in calcium-free solutions. Eventually, the calcium content of the interior would also be reduced, and then the light should no longer be able to gelate the plasmagel. Actually, experimental evidence which seems to support this notion has been obtained with amoebae immersed in pure solutions of potassium and sodium chloride. Light can gelate the plasmagel of amoebae that have been immersed in pure potassium or sodium chloride solutions for short periods of time, during which the potassium or sodium probably replaces only the calcium in the cortex of the amoebae. However, when the amoebae have been in either of these two solutions long enough for most or all of their calcium to be replaced, light is ineffective in gelating the plasmagel. Moreover, it is interesting to note that ammonium oxalate solution, which presumably is much more efficient than potassium or sodium chloride solutions in reducing the calcium content of amoebae, is much more efficient in preventing the gelating action of light on the plasmagel. Thus it seems that there is a source of calcium in the interior of amoebae which persists for some time after calcium in the cortical protoplasm has been replaced. It is possible, of course, to propose alternative theories, and the gelation of the plasmagel of amoebae may be dependent on factors other than calcium; but, as far as these experiments go, they indicate that calcium is involved in one way or another. At present there is no definite evidence as to the nature of any calcium combination in the interior of an amoeba that might be affected by light. It may well be that one of the many types of crystals which can be seen in the protoplasm of amoebae (cf. Pollack, 1928; and Schewiakoff, 1893) is a source of calcium.

Some of the results presented in the body of this paper show clearly that photody-

namic action tends to liquefy the protoplasm in the interior, as well as that at the surface, of amoebae. These liquefying effects cannot be due to light alone or to the dye solutions alone, since, as has been shown, light causes gelation of the two protoplasmic regions of the amoeba and since the dye solutions used in these experiments have practically no effect, in the dark, on the viscosity of amoeba protoplasm. This being true, it becomes a question of just how the combined action of light and rose bengal or eosin can effect a liquefaction of amoeba protoplasm. According to the calcium-release theory, a liquefaction of the plasmagel indicates a release of calcium from a cortical gel or proteinate, whereas a liquefaction of the plasmasol indicates that only small amounts of the calcium released by the stimulant is reaching the interior of the amoeba. In view of the fact that phthalein dyes, like rose bengal and eosin, have a great affinity for proteins (see Rosenthal, 1926), it appears feasible that the dyes could combine with a cortical calcium proteinate and thus sensitize it to light. Exposure to light might then result in the release of calcium from the proteinate, with subsequent liquefaction of the plasmagel. But, what about the liquefying effect of photodynamic action on the plasmasol? Here the main question is whether or not in the light the dyes pass into the cell interior. If the dyes do not get any farther than the cortex, some of the dye molecules—those not already combined with protein—may form calcium bengalate or eosinate with the calcium that has been released from any part of the cell. Thus, only a small amount of calcium would enter or remain free in the interior of the amoeba, and this would result in a liquefaction of the plasmasol (see Heilbrunn, 1928). On the other hand, if the dye molecules readily enter amoeba protoplasm under the influence of light, they might keep the free calcium content of the plasmasol low by tying up most of the calcium as calcium bengalate or eosinate, even though considerable amounts of calcium had been released. Obviously, the validity of either of these proposed mechanisms for photodynamic action on the viscosity of amoeba protoplasm depends upon the ability of calcium to combine with dyes under such conditions as exist in amoeba protoplasm. Although any definite proof of this point must come from future experiments, it should be noted that acid phthalein dyes do combine with calcium (see Beacall, 1926). In any case, my results indicate that only a small amount of any calcium released in amoebae by photodynamic action remains free to produce changes in the viscosity of the protoplasm.

As was mentioned above, the experiments on amoebae represent the first direct study of the effects of photodynamic action on the viscosity of protoplasm. Obviously, the question as to whether photodynamic action in general has a liquefying effect on the physical state of protoplasm can be answered only after more such direct studies have been made. However, some experiments done on the unfertilized eggs of *N. limbata* indicate a negative answer to this question. Citrates and oxalates, which presumably remove calcium from the cortex as well as bind free calcium inside the eggs, tend to prevent photodynamic action and other stimulating agents from producing germinal-vesicle breakdown in the eggs (see Alsup, 1941; Heilbrunn and Wilbur, 1937; and Wilbur, 1941). In other words, photodynamic action seems to produce germinal-vesicle breakdown only when the calcium content of the cortex has not been greatly reduced and when most of the calcium released to the interior of the egg remains free. Such a penetration of free calcium to the interior of the egg would, on the basis of the calcium-release theory, entail a gelation of the internal protoplasm—a situation that would be directly opposed to that which seems to obtain for amoeba protoplasm. Thus it seems probable that photo-

dynamic action can either increase or decrease the viscosity of protoplasm, depending on which living system is involved.

Should viscosity studies show that photodynamic action actually does gelate the protoplasm of such living systems as marine eggs, the mechanism involved would not necessarily have to be different from one of those proposed in this paper for the liquefying action of light-activated dye molecules on amoeba protoplasm. Activated dye molecules which seem to tie up free calcium might act on marine eggs in much the same way that dilute solutions of citrates and oxalates do. Wilbur (1941) found that solutions of the latter two calcium-binding substances can produce germinal-vesicle breakdown as well as prevent its production by stimulating agents. Thus, substances which remove calcium from protoplasm and prevent stimulation (gelation of internal protoplasm) can, under some conditions, act as stimulating agents, i.e., cause gelation of internal protoplasm. At present no satisfactory explanation is available for this twofold action of citrates and oxalates. In the case of photodynamic action it might merely be a matter of the degree to which activated dye molecules penetrated the protoplasm. Gelation of internal protoplasm by photodynamic action might mean that few activated dye molecules were entering the cortex or interior of the cells, and thus few of them would be available to tie up any free calcium in the cells. At any rate, it must be mentioned again that more investigations are needed before any of these suggestions as to the relationship of activated dye molecules to free calcium can be accepted.

It has frequently been pointed out that the colloidal behavior of protoplasm is similar to the colloidal behavior of vertebrate blood (see Heilbrunn, 1937, 1940); and, indeed, in both cases calcium is intimately concerned with the mechanism of gelation or clotting. It is interesting to note that the liquefying effect of photodynamic action on amoeba protoplasm finds a parallel in photodynamic studies of blood. Howell (1921) and Sellards (1918) find that blood-clotting is prevented by photodynamic dyes in the presence of light.

## SUMMARY

1. Centrifuge tests on *A. proteus* show that intense artificial illumination increases the viscosity of the plasmagel approximately 30 per cent. This increase in viscosity can be detected within 10 seconds after the exposure and persists for over 15 minutes.

2. Calcium is essential for this gelating action, since light does not increase the viscosity of the plasmagel of oxalated amoebae.

3. In pure solutions of potassium or sodium chloride the effect of light depends upon the period of immersion. After relatively short immersions light increases the viscosity of the plasmagel, whereas after long periods it cannot do so. This is interpreted to mean that light can release calcium from a source in the interior of the amoeba after it has been removed from the cortex by the action of these salts.

4. The plasmasol or inner protoplasm of *A. dubia* is also gelated by short exposures to intense visible radiation. This effect can be detected within 10 seconds after the exposure and persists for at least 3 minutes.

5. The gelating action of light on the plasmasol is prevented by the removal of calcium from the amoeba.

6. In strengths of 1:50,000 and 1:10,000 rose bengal has no effect in the dark on the viscosity of either the plasmagel or the plasmasol of amoeba. Eosin in a strength of 1:2,000 also produces, in the dark, no changes in the viscosity of amoeba protoplasm.

7. Light acting in combination with either of these two fluorescent dyes, i.e., photodynamic action, causes a decrease in the viscosity of the plasmagel of about 23 per cent.

8. Photodynamic action causes a pronounced liquefaction of the internal protoplasm or plasmasol of *A. dubia*.

9. All of the liquefying effects of photodynamic action are detectable within 10 seconds after the exposures and last as long as 28 minutes.

10. In view of the fact that calcium in relatively small concentration causes a decrease in protoplasmic viscosity (of the cell interior), it is suggested that the liquefying effects of photodynamic action are due primarily to a binding by light-activated dye molecules of most of the free calcium in amoebae.

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# THE RELATION BETWEEN TEMPERATURE, LIGHT, AND RATE OF LOCOMOTION IN PERANEMA TRICHOPHORUM AND RESPONSE TO CHANGES IN TEMPERATURE[1]

(Five figures)

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IT IS well known that, as the temperature increases, the rate of most biological processes increases from 0 to a maximum and then decreases to 0 (Bělehrádek, 1935, p. 2); but the relation between temperature and rate of locomotion has not been extensively investigated, and no observations concerning this relation have been made on *Peranema*. Before considering this problem it will, however, be expedient to ascertain the effect of time on the rate of locomotion at a given temperature.

## THE RELATION BETWEEN RATE OF LOCOMOTION AND TIME AT DIFFERENT TEMPERATURES

The peranemas used were cultured in 350-cc. fingerbowls, each containing about 10 cc. of tap water, a crushed grain of wheat, bacteria, and chilomonads. The cultures were kept at $22° \pm 2°$ C. in diffused daylight.

A Pitts (1932) temperature apparatus mounted on the stage of a compound microscope was used to control the temperature during the experimental observations. Water of a desired temperature was directed, by means of rubber tubes from a thermos jug, through the temperature apparatus, and light from a microscope lamp was reflected, by means of the substage mirror, up through the opening in the temperature apparatus. No substage condenser was used. The light had an intensity of 538-meter candles at the upper surface of the stage of the microscope.

The cover glasses, slides, and pipettes used were cleaned by washing in 95 per cent alcohol and rinsing in distilled water.

The observations were made as follows: A peranema was transferred from a vigorous culture to a few drops of fluid from this culture which had passed through a porcelain filter, then transferred to another drop of this filtered fluid, and finally put into a third drop on a thoroughly cleaned cover glass. This cover glass was then inverted, placed over the opening in the bottom of the metal cup in the temperature apparatus, and sealed with vaseline. Another cover glass was then placed over this opening on the outside and sealed with vaseline. The water containing the peranemas between the two cover glasses spread out over an area about 4 mm. in diameter. (Peranemas thus treated and mounted will hereafter be referred to as "mounted specimens.")

After the peranema was mounted, the metal cup was placed in the temperature apparatus and the light turned on. Then the temperature was changed at the rate of 3° per minute from room temperature ($22° \pm 2°$ C.) to that desired for observation and main-

[1] I wish to express my appreciation to Professor S. O. Mast, who suggested the problem, directed the experimental observations, and made valuable suggestions concerning the preparation of the manuscript.

tained at that temperature to within $\pm 0.^{\circ}5$ for 10 minutes, after which the rate of locomotion was measured in the following manner.

By means of a camera lucida the image of the peranema was projected onto a sheet of black paper. Then as the peranema moved across the field of the microscope the path of the projected image was traced with a white pencil, as long as movement directly forward continued, and the time measured by means of a stop watch. This was then repeated until three paths had been traced every 20 minutes for 1 or 2 hours. If locomotion continued for more than 2 hours, the observations on this specimen were discontinued. Then the length of each of the paths was measured and the average rate of locomotion calculated. The whole process was then repeated with other specimens at this temperature and at other selected temperatures. The results obtained are presented in Table 1.

TABLE 1*

THE RELATION BETWEEN RATE OF LOCOMOTION AND TIME AT DIFFERENT TEMPERATURES, RANGING FROM 14° TO 40°

| Time in Minutes | Rate of Locomotion in μ per Second | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14° | 16° | 18° | 22° | 26° | 28° | 32° | 34° | 36° | 38° | 40° |
| 0 | 13.65 | 18.8 | 21.8 | 29.67 | 40.29 | 41.21 | 29.1 | 26.06 | 53.7 | 45.0 | 45.45 |
| 20 | 13.03 | 20.6 | 25.7 | 32.72 | 35.45 | 41.81 | 43.0 | 38.8 | 54.7 | 35.2 | 21.21 |
| 40 | 15.45 | 20.6 | 25.7 | 36.36 | 41.81 | 38.18 | ..... | 45.45 | 58.5 | 37.4 | 00.00 |
| 60 | 14.2 | 16.4 | 25.4 | 35.15 | 37.76 | 41.2 | 49.7 | 44.8 | 55.1 | 32.9 | 00.00 |
| 80 | ..... | 20.0 | 27.8 | 34.5 | ..... | 43.02 | 37.5 | 39.99 | 54.5 | 00.0 | ..... |
| 100 | ..... | 21.8 | 27.8 | 34.54 | ..... | ..... | 46.6 | 47.27 | ..... | ..... | ..... |
| 120 | ..... | 21.2 | 29.0 | 36.36 | ..... | ..... | 47.3 | 34.54 | ..... | ..... | ..... |

* Nearly all the rates of locomotion are the averages of three paths made by one peranema.

Table 1 indicates that in constant temperatures between 14° and 36° the rate of locomotion in *Peranema* is fairly constant for at least 2 hours but that there is more variation in the temperatures which are above room temperature than in those which are below and that, at 38°, and above, it decreases rapidly with time.

The results obtained in this investigation agree, essentially, with what has been found for various biological processes in other organisms, for example, Greeley (1902) on activity in *Stentor;* Buglia (1911) on contraction in the esophagus of the chick; Ishikawa (1913) on locomotion in *Amoeba limax;* Bělehrádek (1935) on action of the heart in *Daphnia;* and Michelson (1928) on ciliary action in *Paramecium.*

## THE RELATION BETWEEN TEMPERATURE AND THE RATE OF LOCOMOTION

The peranemas used were cultured, washed, and mounted as described above, except that four were mounted in each metal cup. One of the metal cups was then put into the Pitts temperature apparatus at room temperature ($22° \pm 2°$ C.). This was then changed to the selected temperature at the rate of 3° per minute and maintained at this temperature to within $\pm 0.^{\circ}5$ for 10 minutes, after which ten paths of one of the peranemas, selected at random, were traced as described above. Then ten more paths were obtained with each of six other groups of four peranemas. The length of each of the paths was

then measured and the rate of locomotion calculated. The whole process was then repeated with other peranemas from the culture, exposed to different temperatures. In all, paths of over three hundred peranemas in one hundred mounts were measured. The results obtained are presented in Table 2 and Figure 1.

TABLE 2*

RELATION BETWEEN TEMPERATURE AND RATE OF LOCOMOTION IN *P. trichophorum*

| Temperature (° C.) | Average Length of Paths ($\mu$) | Average Time in Seconds | Average Rate of Locomotion ($\mu$ per Second) | Standard Deviation | Probable Error | $Q_{10}$ | $\mu$ |
|---|---|---|---|---|---|---|---|
| 10 | 290.80 | 29.86 | 9.75 | 2.22 | 0.18 | | |
| | | | | | | 2.23 | |
| 12 | 423.27 | 36.95 | 11.45 | 2.52 | 0.20 | | |
| | | | | | | 3.78 | |
| 14 | 713.40 | 47.86 | 14.90 | 3.62 | 0.29 | | |
| | | | | | | 4.23 | 19,320 |
| 16 | 699.10 | 35.16 | 19.88 | 3.96 | 0.32 | | |
| | | | | | | 3.96 | |
| 18 | 834.20 | 31.85 | 26.18 | 5.06 | 0.41 | | |
| | | | | | | 0.97 | |
| 20 | 827.69 | 31.77 | 26.05 | 6.12 | 0.47 | | |
| | | | | | | 3.20 | |
| 22 | 891.57 | 27.69 | 32.19 | 6.45 | 0.52 | | |
| | | | | | | 1.75 | |
| 24 | 777.98 | 21.15 | 36.79 | 5.26 | 0.43 | | |
| | | | | | | 1.13 | |
| 26 | 841.15 | 22.33 | 37.67 | 8.78 | 0.71 | | |
| | | | | | | 1.69 | |
| 28 | 839.40 | 20.05 | 41.87 | 7.94 | 0.65 | | 5,672 |
| | | | | | | 1.49 | |
| 30 | 850.72 | 18.74 | 45.39 | 6.18 | 0.50 | | |
| | | | | | | 0.83 | |
| 32 | 841.88 | 19.26 | 43.71 | 7.03 | 0.57 | | |
| | | | | | | 1.06 | |
| 34 | 864.69 | 19.51 | 44.32 | 8.36 | 0.68 | | |
| | | | | | | 2.44 | |
| 36 | 929.09 | 17.55 | 52.94 | 9.43 | 0.89 | | |
| | | | | | | 0.27 | |
| 38 | 788.14 | 18.40 | 40.66 | 7.33 | 0.59 | | −16,560 |
| | | | | | | 0.67 | |
| 40 | 782.39 | 20.76 | 37.69 | 10.66 | 0.86 | | |
| 42 | ..... | ..... | 0.0 | ..... | ..... | ..... | −∞ |

* Approximately seventy paths were measured for each temperature in the table. The values of $\mu$ were obtained by calculations based on the straight lines in Figure 2.

Figure 1 indicates that, as the temperature increased, the average rate of locomotion increased from 9.75 $\mu$ per second at 10° to a maximum of 45.38 at 30°, then decreased slightly, then increased to a second maximum of 52.94 $\mu$ per second at 36° and then decreased rapidly to 0 at 42° (at 42° and above, the peranemas round up and the flagellum lashes slowly, but there is no locomotion). The curves for maximum and minimum rates of locomotion also indicate a double maximum in the region of 30°–36°.

Table 2 shows, however, that the probable error for the average rate of locomotion is

so large in this region that the observed differences in the average rates of locomotion are probably not statistically significant. There is consequently no real evidence in support of the double maximum.

Schwitalla (1924*a* and *b*) and Mast and Prosser (1932) in observations on *Amoeba proteus* maintain that, as the temperature is increased, the rate of locomotion increases to a maximum at about 24° C., then decreases, and then increases again to a second maximum at 30° C. Hopkins (1937) in observations on a marine amoeba found that if the temperature increases rapidly there are two maxima but that if it increases slowly there is only one. There is, however, no evidence that the difference between the maxima obtained in any of these investigations is statistically significant.

The values of the temperature coefficient ($Q_{10}$ in the van't Hoff equation)[2] and the temperature characteristic ($\mu$ in the Arrhenius equation)[3] were calculated for the average rates of locomotion at different temperatures. These values are presented in Table 2, and the logarithm of the rate of locomotion is plotted against the reciprocal of the absolute temperature in Figure 2.

Table 2 shows that the value of $Q_{10}$ is clearly much higher for the lower than for the higher temperatures. This indicates that the processes associated with locomotion in *Peranema* vary with the temperature.

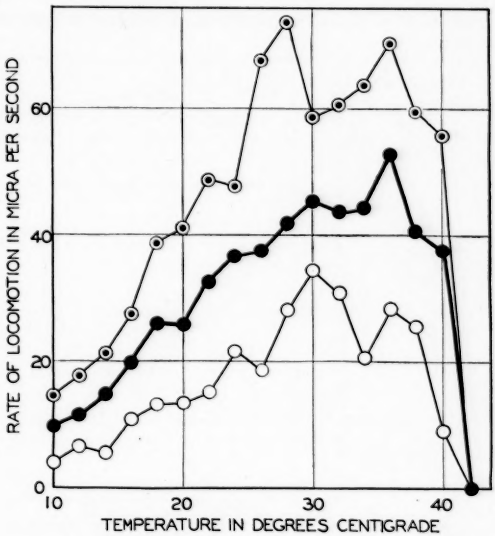


FIG. 1.—Relation between temperature and rate of locomotion in *P. trichophorum*. ●, average rate of locomotion; ⊙, highest rate of locomotion; ○, lowest rate of locomotion. Each point on the curve for the average rate of locomotion represents the measurement of seventy paths.

Figure 2 shows that the line produced by plotting the logarithm of the average rate of locomotion of *Peranema* at different temperatures against the reciprocal of the absolute

[2] The van't Hoff equation:

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{10/(t_2-t_1)},$$

where $K_1$ and $K_2$ are rates of locomotion at temperatures $t_1$ and $t_2$, respectively.

[3] The Arrhenius equation:

$$K_2 = K_1 \cdot e^{\left[\frac{\mu}{2}\left(\frac{1}{T_1}-\frac{1}{T_2}\right)\right]}; \qquad \mu = \frac{4.6\,(\log K_2 - \log K_1)}{\frac{1}{T_1} - \frac{1}{T_2}},$$

where $K_1$ and $K_2$ are rates of biological processes at absolute temperatures $T_1$ and $T_2$, respectively, and $e$ is the basis for natural logarithms. The value of $\mu$ is assumed to express the number of calories necessary to change 1 gm. equivalent of inactive molecules into active ones.

temperature is continuously curved. This indicates that as the temperature changes there are no abrupt changes in the processes governing the rate of locomotion in *Peranema*. The fact, however, that the value of $\mu$ varies enormously with the temperature—being approximately 19,320 calories for 10°–20°; 5,672 for 22°–36°; −16,560 for 36°–40°; and $-\infty$ for 40°–42°—shows that while there appear to be no abrupt changes in these processes they differ greatly in different temperatures over the range tested.

The results obtained in this investigation agree with those obtained by others in that they indicate differences in the processes involved in the regulation of the rate of locomotion at different temperatures but not in reference to the rate of change in these proc-

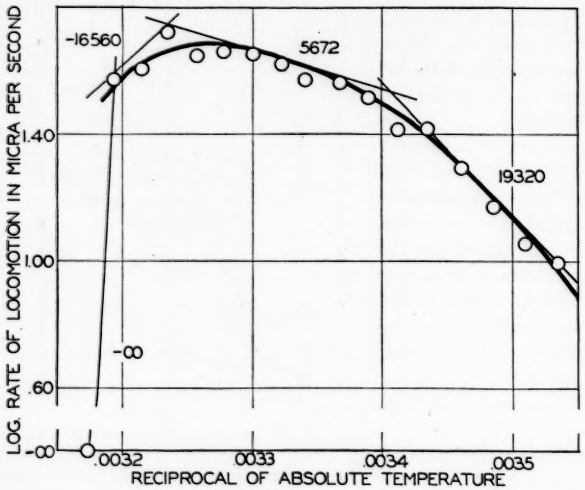


FIG. 2.—The relation between the reciprocal of the absolute temperature and the logarithm of the rate of locomotion in *P. trichophorum*.

esses with changes in temperature—for example, Shapley (1920) on the ant, *Liometopum apiculatum* Mayr; Glaser (1924) on *Paramecium caudatum;* Crozier (1924*a*) on a diplopod; Cole (1926) on *Planaria;* Miller (1928) on the blowfly larva, *Lucilia sericata* Meigen; and Welsh (1932) on the mussel crab, *Pinnotheres maculatus* Say.

## THE EFFECT OF ADAPTATION TO A GIVEN TEMPERATURE ON THE RATE OF LOCOMOTION AT ANOTHER TEMPERATURE

Hopkins (1937) found that the rate of locomotion in a marine amoeba, at a given temperature, depends upon the temperature to which it has previously been adapted. The question, therefore, arises as to whether this also obtains for *Peranema trichophorum*. This question is considered in the following pages.

Peranemas were taken from cultures kept at 22° ± 2° C., washed, and mounted as described above. The mounted peranemas in the metal cups were then put into temperature-control boxes at 12° ± 0°.5 and left at least 24 hours, then one of the cups containing two or three peranemas was put into the Pitts temperature apparatus at 12° C. The

temperature was then changed at the rate of 2°–3° per minute to 14°, after which three paths of a peranema, selected at random, were traced as described above. This was then repeated with observations on the rate of locomotion at the same and other temperatures until six paths for each temperature were in hand. (In some preparations only one peranema was active, and all observations were made on it.) Then the rate of locomotion at each temperature was calculated as described above. The whole process was then repeated with peranemas adapted to 32° in place of 12°. The results obtained are presented in Figure 3.

Figures 3 indicates that in all the temperatures between 14° and 38° the rate of locomotion in *P. trichophorum* was higher for specimens which had previously been adapted to 12° than for those which had been adapted to 32°, that at 38° it was practically the same, and that at 40° it was slightly lower.

It can therefore be concluded that the rate of locomotion in *Peranema* at any given temperature depends upon the temperature to which it had previously been adapted.

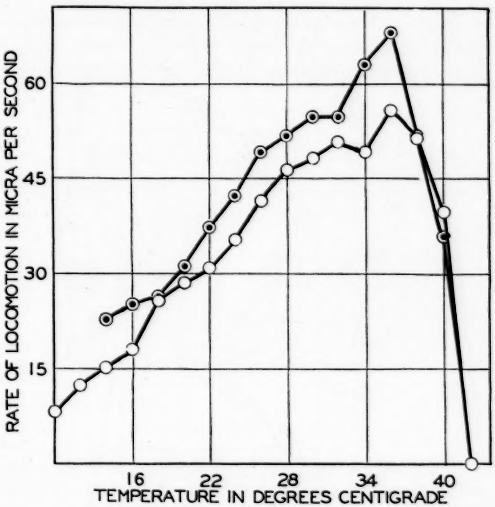


FIG. 3.—The rate of locomotion of *P. trichophorum* at different temperatures after adaptation for 24 hours to 12° and 32°, respectively. ⊙, 12°; ○, 32°. Each point on the curves represents the average rate of locomotion of six paths, three for each of two peranemas.

### RELATION BETWEEN RATE OF LOCOMOTION, TEMPERATURE, AND LIGHT

The relation between light intensity and rate of locomotion has been investigated for a considerable number of different organisms, but the relation between rate of locomotion, temperature, and light of various intensities has not been studied in any. The present investigation concerns this relationship in *P. trichophorum*.

The peranemas used were cultured, washed, and mounted as described above. A Pitts temperature apparatus was used to regulate the temperature. A 1,000-watt monoplane stereopticon bulb mounted in a box, light-tight except for an opening 2.5 cm. square, and lenses from a projection lantern were used to produce the light intensities desired.

A microscope was mounted on a horizontal track parallel with the beam of light from the 2.5-cm. opening in the box, so that this light was reflected by the substage mirror up through the opening in the microscope stage. No substage lens was used. The light on the microscope stage was measured with a Weston photometer.

The observations were made as follows: A number of peranemas were washed in the usual manner and mounted in the metal cups (five specimens to a cup). These were kept

in a darkroom at 24° C. for at least 1 hour. The microscope was placed so that the beam of light was reflected up through the stage and the desired luminous intensity obtained. The temperature apparatus containing the peranemas was then fastened to the stage of the microscope, and the desired temperature was obtained and maintained within a variation of ±0°.5 by running water through it. After 10 minutes with the light turned on, the path of one of the peranemas was traced and the rate of locomotion calculated as described above. This was then repeated with each of the other specimens in the cup, selected at random. Then the whole process was repeated with specimens in other cups at different temperatures and in different luminous intensities. Some of the results obtained are presented in Figure 4.

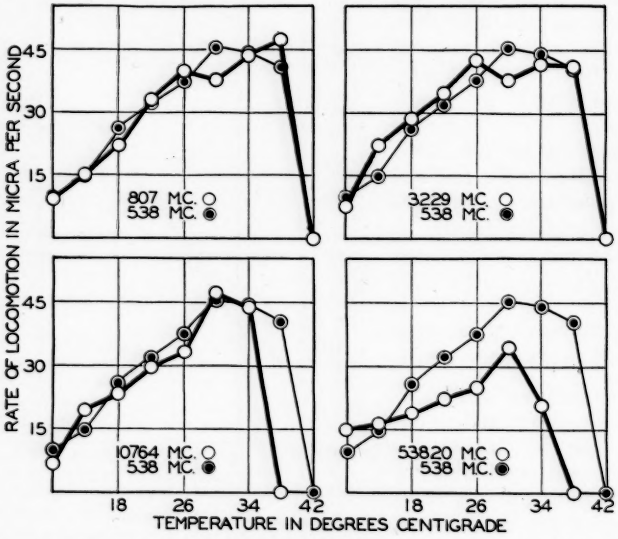


FIG. 4.—The relation between temperature and rate of locomotion of *P. trichophorum* at different luminous intensities. Each point on the heavy curves represents the average rate of locomotion in five paths of about 850 μ for each of five peranemas. Each point on the light-curves represents the average in numerous paths in 538-meter candles (Table 2).

Figure 4 shows that the rate of locomotion in *Peranama* in temperatures between 10° and 40° is essentially the same in luminous intensities of 538-, 807-, and 3,229-meter candles,[4] that in 10,764-meter candles it is essentially the same as this in temperatures between 10° and 34° but much lower at 38°, and that in 53,820-meter candles it is considerably higher than this at 10° but much lower in temperatures between 18° and 38° C.

The results obtained indicate that light of low or moderate intensity has no effect on the rate of locomotion in *Peranema* but that light of high intensity reduces it in temperatures above 14° and increases it in temperatures below 14°.

Weber (1929) found that strong light increases the viscosity of the protoplasm in the

[4] The rate of locomotion in 1,614- and 5,382-meter candles, respectively, was also measured. The results obtained are practically the same as those obtained in 807- and 3,229-meter candles, respectively.

guard cells of *Vicia*. Mast (1932) found that it produces increase in thickness of the plasmagel sheath in *Amoeba*, and Mast and Stahler (1937) demonstrated that it causes marked decrease in rate of locomotion in this animal. The decrease in rate of locomotion observed in *Peranema* in high intensities and above 14° C. is therefore probably due to increase in viscosity of the cytoplasm. The increase in rate of locomotion below 14° C. in high light intensities is probably due to increase in internal temperature caused by absorptions of light by the cytoplasm.

### RESPONSE OF *P. trichophorum* TO RAPID CHANGES IN TEMPERATURE

Mast (1912, Fig. 1) and Mast and Hawk (1936) found that *P. trichophorum* responds to contact and to rapid increase in luminous intensity by a sharp deflection of the anterior end. The question therefore arises as to whether or not rapid changes in temperature induce similar responses. This question and others are considered in the following pages.

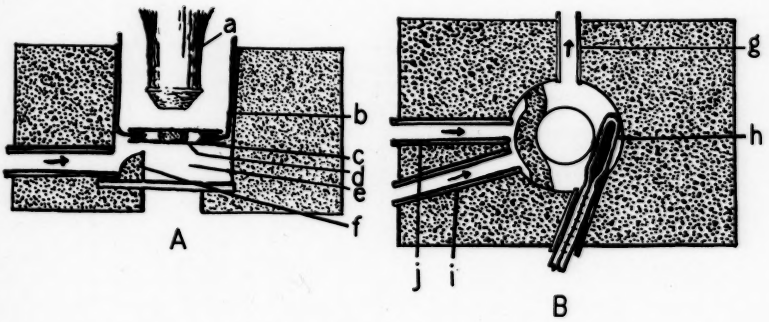


FIG. 5.—Modified Pitts temperature apparatus used to produce rapid changes in temperature. *A*, cross-section; *B*, horizontal section; *a*, objective; *b*, metal cup; *c*, vaseline seal; *d*, drop of water; *e*, water chamber; *f*, baffle ridge; *g*, outlet; *h*, thermometer; *i*, warm-water inlet; *j*, cold-water inlet.

A Pitts temperature apparatus was modified as follows: The space around the cup was reduced, and two water inlets were made, one for each of two temperatures (Fig. 5). The thermometer was inserted into the chamber of the temperature apparatus until the bulb was close to the metal cup. Two thermos jugs, one containing water at room temperature ($22° \pm 2°$ C.) and the other at a much lower or a much higher temperature, were connected with the two inlets by means of rubber tubes. Across the two inlet tubes a rocking cutoff switch was fastened close to the temperature apparatus. By throwing the switch, the flow from one thermos jug was suddenly stopped and that from the other started.

A by-pass outlet was provided near the switch so that hot or cold water could be maintained in the tube leading from the jug to the switch. By this means hot or cold water was brought to the temperature apparatus very quickly after the switch was thrown. If the temperature was to be raised, water of about 80° C. was put into one of the jugs. If it was to be lowered, a mixture of ice and water was used. The temperature in the cup, therefore, changed rapidly after the switch was thrown, i.e., $0.°3$–$1.°5$ per second.

The observations were made as follows: Four peranemas were mounted in a cup as

described above. The cup was then put into the temperature apparatus with the microscope lamp turned on and water at room temperature flowing through the apparatus. The temperature was then rapidly increased or decreased by throwing the water switch and the action of the peranemas noted.

Peranemas deflect the anterior end at irregular intervals without any environmental changes. It is consequently possible to have this deflection occur coincidentally with the change in temperature without being caused by it. To avoid falling into error owing to this, we observed all the peranemas in the cup, and only if the anterior end of all deflected at the same time after the change in temperature was it concluded that there was a response.

TABLE 3*

RELATION BETWEEN RATE AND EXTENT OF INCREASE IN TEMPERATURE AND RESPONSE IN *P. trichophorum*

| Designation of Tests | Temperature at Beginning of Rapid Change (° C.) | Increase in Temperature (° C.) | Time Required (Seconds) | Rate of Increase (° per Second) | Reaction Time (Seconds) | Recovery Period (Seconds) |
|---|---|---|---|---|---|---|
| 1 | 23.5 | 1.5 | 6.0 | 0.25 | No response | |
| 2 | 24.0 | 2.5 | 7.5 | 0.33 | | |
| 3 | 24.5 | 3.5 | 9.0 | 0.39 | | |
| 4 | 24.0 | 7.0 | 10.0 | 0.70 | 13.00 | 40.0 |
| 5 | 22.2 | 8.2 | 7.98 | 1.03 | 13.52 | 29.8 |
| 6 | 23.5 | 9.0 | 7.75 | 1.15 | 11.75 | 40.0 |
| 7 | 19.8 | 13.9 | 8.90 | 1.55 | 12.75 | 32.5 |
| 8 | 24.0 | 10.0 | 7.00 | 1.43 | 10.60 | 59.0 |
| 9 | 24.0 | 7.5 | 5.13 | 1.45 | 7.89 | 21.2 |

* The reaction time is the time in seconds from the beginning of increase in temperature to response. The recovery period is time from response until movement begins again.

It was found that, if the temperature is rapidly increased or decreased, all the peranemas in the cup respond by bending sharply, precisely as they do if the luminous intensity is rapidly increased, but that if it is slowly increased or decreased this response does not take place.

These results are similar to those obtained by Ewart (1903) and Cook (1929) in observations on the effect of rapid changes in temperature on protoplasmic streaming in *Chara* and *Nitella*.

Details concerning this response were investigated as follows: Two persons with stop watches took part in the experiment. One regulated the flow of water through the temperature apparatus and controlled the temperature, the other observed the peranemas.

Peranemas mounted in the metal cup in the temperature apparatus as described above were left for 3 minutes at moderate temperature, i.e., until they were moving normally; then the switch was thrown, admitting hot water from the jug, but for only a few seconds; then the switch was thrown again, admitting water of room temperature. During the time the hot water was on, the temperature in the cup increased rapidly, and after it was turned off it decreased very slowly.

Both stop watches were started immediately after the temperature in the cup began

to rise. By manipulating these, the time during which the temperature in the cup increased and the intervals between the beginning of increase in temperature and response (reaction time) and from the response until locomotion was resumed (recovery period) were measured. The change in temperature was measured by means of the thermometer in the cup.

This was repeated with specimens in other cups subjected to different rates of increase in temperature, after which the whole process was repeated with specimens subjected to rapid decrease in temperature in place of increase.

The results obtained are presented in Tables 3 and 4. These tables show that with rapid increase in temperature the reaction time varied from 7.89 to 13.52 seconds and

TABLE 4*

RELATION BETWEEN RATE AND EXTENT OF DECREASE IN TEMPERATURE AND RESPONSE IN *P. trichophorum*

| Designation of Tests | Temperature at Beginning of Rapid Change (° C.) | Decrease in Temperature (° C.) | Time Required (Seconds) | Rate of Decrease (° per Second) | Reaction Time | Recovery Period |
|---|---|---|---|---|---|---|
| 1 | 21.7 | 2.0 | 6.6 | 0.30 | No response | |
| 2 | 21.5 | 2.5 | 7.5 | 0.33 | | |
| 3 | 21.5 | 3.0 | 7.8 | 0.38 | | |
| 4 | 22.0 | 3.3 | 8.16 | 0.40 | 11.9 | 31.7 |
| 5 | 25.3 | 5.3 | 8.10 | 0.65 | 12.6 | 28.6 |
| 6 | 24.1 | 5.1 | 7.00 | 0.75 | 14.5 | 39.8 |
| 7 | 23.0 | 3.5 | 5.00 | 0.70 | 10.3 | 15.2 |
| 8 | 23.0 | 4.0 | 5.00 | 0.80 | 9.1 | 15.8 |
| 9 | 21.5 | 4.5 | 5.00 | 0.90 | 10.0 | 15.5 |

*The reaction time is the time in seconds from the beginning of decrease in temperature to response. The recovery period is time from response until movement begins again.

the recovery period from 59.0 to 21.2 seconds and that with decrease in temperature the reaction time varied from 9.1 to 14.5 seconds and the recovery period from 15.2 to 39.8 seconds but that these variations were independent of the respective rate of change in temperature.

The fact that, in *Peranema*, changes in light intensity and in temperature and in contact produce the same response indicate that the internal processes involved in this response are primarily physical rather than chemical, for it is difficult to see how these three stimulating agents could induce the same chemical changes in the organisms, whereas it can readily be seen how they could induce the same physical changes and consequently the same response.

Folger (1924) and Mast (1936) reached the same conclusion regarding response in *A. proteus* to mechanical agitation and photic stimulation. Mast says in reference to this:

> It is well known that light produces photoelectric effects which may consist in changes in position of the valency electrons in the molecules . . . . and that this results in the activation of the molecules. It is consequently evident that light can produce changes in the electric equilibrium in the molecules . . . . and it is quite conceivable that this could cause an alignment of the

molecules so as to result in the formation of a gel. It is also well known that mechanical agitation facilitates the alignment of the molecules in the process of crystallization. This seems to indicate that it could in some way facilitate gelation. If this is true, it supports Folger's contention that stimulation by light is due primarily to photo-physical effects.

## SUMMARY

1. The rate of locomotion in *Peranema* varies but little with time in constant temperatures ranging from 14° to 36°, but it decreases rapidly in temperatures higher than 36° C.

2. As the temperature increases, the rate of locomotion increases from $9.75 \pm 0.18$ $\mu$ per second at 10° to a maximum of $45.39 \pm 0.50$ $\mu$ at 30°, then perhaps decreases slightly and increases to a second maximum of $53.94 \pm 0.89$ $\mu$ at about 36° C. and then decreases rapidly to zero at 43°.

3. The temperature coefficient ($Q_{10}$ in the van't Hoff equation) decreases from 2.23 $\mu$ at 10° to 0.67 $\mu$ at 40°.

4. The value of $\mu$ in the Arrhenius equation is about 19,320 for 10°–20°, 5,672 for 22°–36°, −16,560 for 36°–40°, and $-\infty$ for 40°–42°, but the change in these values is not abrupt. This indicates that the processes involved in regulating the rate of locomotion differ greatly in different temperatures but that there are no critical temperatures.

5. The rate of locomotion at any given temperature depends upon the temperature to which the peranemas had previously been adapted.

6. Light of moderate intensity has no appreciable effect on the rate of locomotion in *Peranema* in any temperature, but very intense light causes considerable increase in the rate of locomotion in temperatures below 14° and marked decrease in temperatures above 14° C.

7. If the temperature is rapidly and extensively increased or decreased, *Peranema* responds by a sharp deflection of the anterior end; but if the temperature is slowly changed this response does not occur. This response is the same as that given to contact and to rapid changes in light intensity. It is probably due primarily to physical rather than to chemical changes in the protoplasm.

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# FECUNDITY, LONGEVITY, AND PARTHENOGENESIS OF THE AMERICAN ROACH, PERIPLANETA AMERICANA L.[1]

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ALTHOUGH the American cockroach, *Periplaneta americana*, is among the commoner insect pests of homes, stores, and buildings, much of its biology has not been ascertained; and not all available data are in agreement. The purposes of the experiments reported here were to study the egg-laying capacity of the laboratory-reared American roach in the presence or absence of a male and to obtain information about the number of eggs per capsule, the incubation period of eggs, parthenogenesis, longevity, and other allied phenomena. Such knowledge is valuable in view of recent increasing use of the cockroach as an experimental animal in insect physiology and toxicology. This paper presents results obtained from 85 females, some of which were observed over a period of more than 2 years.

Von Fischer (1928) in her study of development of the American roach reported that Fibiger had found the incubation period for eggs of the same species to be 6 weeks; and nymphal stages to extend over a period of 7–8 months. She stated that Fibiger's notations for the developmental period were too long, since she observed that the imago emerged after about 5 months. Sein (1923) also obtained an incubation period of slightly more than 6 weeks for eggs of *P. americana*.

Several papers have originated from observations of specimens of this roach in Egypt and India. Nigam (1933) investigated oviposition and development of *P. americana* in India. He was interested mainly in the general biology of the insect but stated that oviposition usually begins 3–7 days after copulation. The incubation period for eggs was reported to be 27–28 days. He noted that egg capsules were usually produced during summer months. Parthenogenesis was not observed. Lefroy (1909) gave 23 days as the duration of incubation; while Adair (1923) found 29 days as a minimum and 64 days as a maximum for the time between oviposition and hatching.

Gould and Deay (1938) published a biological study of *P. americana*, which was primarily concerned with oviposition and longevity. From observations upon 14 selected individuals they found that adulthood of females varied from 102 to 588 days and averaged 438 days. An average of 58.8 egg cases was produced per female, with individual production ranging from 10 to as many as 90 capsules. Egg cases averaged 5.9 days apart; and 13.8 nymphs hatched per capsule. Although 3 virgin females were included in the series, none produced viable eggs. Two females had males present only during the preoviposition period, and formed 30 and 40 egg capsules, respectively. On the average, they appeared 7.3 days apart and most of the eggs were fertile.

Rau (1940) published a series of papers concerned with various biological phases of the American roach. He reported that roaches in confinement throughout their entire life-

[1] Journal Paper No. J-928 of the Iowa Agricultural Experiment Station, Ames, Iowa (Project No. 649).

span lived from $8\frac{2}{3}$ to $14\frac{1}{2}$ months as adults. For those confined as late instar nymphs and throughout adulthood, he found adult life-spans of 60–170 days for 7 females, and 30–180 days for 21 males. His results on oviposition were obtained from females of unknown age. They produced an average of 11.9 egg cases, which were formed from 2 to 53 days apart, with an average period of 7.4 days between capsules.

## MATERIALS AND METHODS

Specimens of the American roach, *P. americana*, were used in these experiments. They had been hatched in the laboratory from eggs taken from stock cultures, which have been laboratory reared for at least ten generations. After hatching they were maintained throughout their entire lives upon a diet of whole wheat bread, banana, and raw beef. Only those roaches which lived for 90 or more days as adults were considered in the final summary of data. Thus, females were observed over a period sufficiently long to obtain significant oviposition data; and longevity records are slightly weighted because of exclusion of those females or males which died during the first 3 months of their adult lives. Except for 6 females, all were individually observed throughout their entire imaginal lives. These 6 exceptions were taken from stock cages about 4 weeks after their final molts and probably had mated with adult males in the stock cages before they were isolated for observation.

When the experiments were first set up, either single pairs of roaches or individual females were caged in 1,000-cc. pressure bottles. As the problem expanded, the supply of these bottles became exhausted, and it was necessary to keep individuals and pairs in 250-cc. Erlenmeyer flasks. Since it was felt that restricted activity in smaller chambers might influence the results, data from roaches in the two types of containers were kept separately. Roaches in 1,000-cc. pressure bottles make up Series A; those in 250-cc. Erlenmeyer flasks, Series B.

To determine differences in fecundity of females under various conditions, the following groups were studied:

1. Females with males constantly from the time of molting to adulthood until death (Group I of both Series A and Series B; results tabulated in Tables 1 and 2)
2. Females with males from beginning of adulthood until oviposition began, after which males were never present (Group II of Series A and B; results in Tables 1 and 2)
3. Virgin females isolated from males for from 3 months to more than a year before mating (Group III of Series A; results in Tables 1 and 5)
4. Virgin females isolated from males during their entire adult lives (Group IV of Series B; Table 2)
5. Females with males during only the first 4 weeks of their adult lives (Group V of Series B; Table 6)

Group I was made up of 20 females in Series A; 15 in Series B. Group II contained 10 females in Series A; 9 in Series B. Group III of Series A had 8 females; while Group IV of Series B consisted of 17 virgin females. Group V of Series B included 6 females.

Observations were made daily; and egg cases were removed only when dropped. All experimental animals were kept at laboratory temperature, which varied from 17° to 28° C.

After several methods were tried, the following was adopted as one which seemed to afford optimum conditions for incubation and hatching of egg capsules. Ten-cc. vials,

## TABLE 1

### INDIVIDUAL OVIPOSITION RECORDS FOR FEMALES IN SERIES A, IN 1,000-CC. CONTAINERS

| GROUP I — Males Present throughout Adult Life of Females | | | | | | | GROUP II — Males Present during Preoviposition Period | | | | | | | GROUP III* — Females with Delayed Mating | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Roach Number | Adulthood (Days) | Preoviposition Period (Days) | Production Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case | Roach Number | Adulthood (Days) | Preoviposition Period (Days) | Production Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case | Roach Number | Adulthood (Days) | Preoviposition Period (Days) | Production Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case | Days before Male Added |
| 6C | 398 | 19 | 303 | 36 | 8.4 | 11.1 | 25B | 481 | 18 | 446 | 46 | 9.9 | 10.5 | 16A | 592 | 56 | 260 | 7 | 43.4 | 55.6 | 389 |
| 4B | 364 | 19 | 333 | 40 | 8.5 | 9.1 | 24A | 356 | 15 | 327 | 38 | 8.8 | 9.4 | 22A | 706 | 26 | 142 | 8 | 20.3 | 42.9 | 343 |
| 8C | 344 | 30 | 286 | 33 | 8.9 | 10.4 | 23B | 346 | 18 | 115 | 11 | 11.5 | 31.5 | 29A | 436 | 29 | 296 | 17 | 18.2 | 19.8 | 336 |
| 7B | 298 | 15 | 275 | 52 | 5.4 | 5.7 | 10C | 316 | 6 | 289 | 29 | 10.3 | 10.9 | 27B | 303 | 22 | 1 | 1 | ..... | 208.0 | 208 |
| 2C | 287 | 11 | 268 | 44 | 6.2 | 6.5 | 23A | 278 | 21 | 240 | 12 | 22.4 | 23.2 | 28B | 309 | ..... | ..... | 0 | ..... | ..... | 184 |
| 5B | 264 | 9 | 231 | 34 | 7.0 | 7.8 | 30C | 267 | 17 | 211 | 21 | 10.6 | 12.7 | 46A | 321 | ..... | ..... | 0 | ..... | ..... | 98 |
| 21A | 224 | 15 | 196 | 32 | 6.3 | 7.0 | 31A | 226 | 13 | 206 | 33 | 6.4 | 6.8 | 47A | 367 | 89 | 1 | 1 | ..... | 98 | 98 |
| 1B | 218 | 10 | 202 | 30 | 7.0 | 7.3 | 31B | 197 | 23 | 165 | 20 | 8.7 | 9.9 | 48A | 247 | 65 | 1 | 1 | ..... | 96 | 96 |
| 10B | 215 | 14 | 179 | 37 | 5.0 | 5.8 | 30A | 110 | 15 | 98 | 10 | 9.8 | 11.0 | | | | | | | | |
| 3D | 214 | 16 | 169 | 22 | 8.0 | 9.7 | 49A | 93 | 24 | 63 | 8 | 9.0 | 10.4 | | | | | | | | |
| 4A | 212 | 12 | 198 | 40 | 5.1 | 5.3 | | | | | | | | | | | | | | | |
| 3B | 191 | 15 | 159 | 24 | 6.9 | 8.0 | | | | | | | | | | | | | | | |
| 5A | 191 | 12 | 154 | 32 | 5.0 | 6.0 | | | | | | | | | | | | | | | |
| 1A | 177 | 18 | 63 | 10 | 7.0 | 17.7 | | | | | | | | | | | | | | | |
| 2B | 160 | 14 | 133 | 19 | 7.4 | 8.4 | | | | | | | | | | | | | | | |
| 7A | 155 | 15 | 130 | 24 | 5.7 | 6.5 | | | | | | | | | | | | | | | |
| 6A | 148 | 14 | 126 | 25 | 5.3 | 5.9 | | | | | | | | | | | | | | | |
| 9A | 144 | 21 | 55 | 11 | 5.5 | 13.1 | | | | | | | | | | | | | | | |
| 9B | 142 | 11 | 122 | 19 | 6.2 | 7.5 | | | | | | | | | | | | | | | |
| 8A | 120 | 15 | 77 | 14 | 5.9 | 8.6 | | | | | | | | | | | | | | | |
| Means | 223 | 15.6 | 183 | 28.9 | 6.5 | 8.4 | Means | 267 | 17.0 | 216 | 22.8 | 10.7 | 13.6 | Means | 410 | 47.8 | 117 | 4.4 | 27.3 | 86.7 | ..... |
| Standard deviation | 79.0 | 5.7 | 34.2 | 11.2 | 1.2 | 3.0 | Standard deviation | 117.4 | 5.3 | 49.1 | 13.6 | 4.3 | 7.6 | Standard deviation | 160.1 | 26.7 | 136.7 | 6.0 | ..... | 66.8 | ..... |

* Oviposition data for this Group III are only for the period *prior* to introduction of a male.

## TABLE 2

### INDIVIDUAL OVIPOSITION RECORDS FOR FEMALES IN SERIES B, IN 250-CC. CONTAINERS

| GROUP I — Males Present throughout Adult Life of Females | | | | | | | GROUP II — Males Present during Preoviposition Period | | | | | | | GROUP IV — Virgin Females | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Roach Number | Adulthood (Days) | Preoviposition Period (Days) | Production Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case | Roach Number | Adulthood (Days) | Preoviposition Period (Days) | Production Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case | Roach Number | Adulthood (Days) | Preoviposition Period (Days) | Production Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case |
| 39C | 214 | 100 | 102 | 11 | 10.2 | 19.5 | 11D | 268 | 16 | 249 | 24 | 10.8 | 11.2 | 33B | 327 | 188 | 115 | 9 | 14.4 | 36.3 |
| 35C | 170 | 15 | 39 | 6 | 7.8 | 28.3 | 14B | 246 | 34 | 175 | 26 | 7.0 | 9.5 | 26A | 356 | 28 | 311 | 17 | 19.4 | 27.0 |
| 41C | 133 | 20 | 30 | 5 | 7.5 | 26.6 | 15C | 195 | 73 | 70 | 3 | 23.3 | 65.0 | 17B | 307 | 14 | 288 | 3 | 144.0 | 102.3 |
| 36A | 128 | 34 | 112 | 12 | 8.0 | 10.7 | 13B | 185 | 16 | 141 | 6 | 28.2 | 30.8 | 12B | 294 | 83 | 195 | 15 | 13.9 | 19.6 |
| 39A | 128 | 15 | 109 | 21 | 5.5 | 6.1 | 44C | 177 | 31 | 138 | 5 | 34.5 | 35.4 | 32C | 273 | 22 | 1 | 1 | ..... | 273.0 |
| 43B | 128 | 19 | 97 | 11 | 9.7 | 11.6 | 15B | 133 | 13 | 106 | 7 | 17.7 | 19.0 | 20B | 244 | 14 | 223 | 14 | 17.2 | 17.5 |
| 41A | 126 | 15 | 105 | 21 | 5.3 | 6.0 | 50A | 93 | 24 | 63 | 8 | 9.0 | 10.4 | 20A | 243 | 20 | 187 | 7 | 31.2 | 34.7 |
| 38A | 122 | 24 | 72 | 4 | 18.0 | 30.5 | 18B | 92 | 15 | 27 | 4 | 9.0 | 23.0 | 17A | 230 | 180 | 44 | 5 | 11.0 | 46.0 |
| 36B | 118 | 8 | 90 | 13 | 7.5 | 9.1 | 37D | 91 | 32 | 38 | 6 | 7.6 | 15.2 | 12A | 224 | 22 | 189 | 11 | 18.9 | 20.4 |
| 34B | 113 | 23 | 80 | 10 | 8.9 | 11.3 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 45A | 203 | 21 | 172 | 23 | 7.8 | 8.8 |
| 35A | 107 | 14 | 86 | 11 | 8.6 | 9.7 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 34D | 200 | 21 | 174 | 18 | 10.2 | 11.1 |
| 40D | 103 | 23 | 70 | 13 | 5.8 | 8.0 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 27A | 197 | 128 | 1 | 1 | ..... | 197.0 |
| 40B | 99 | 23 | 64 | 10 | 7.1 | 9.9 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 38B | 185 | 50 | 124 | 10 | 13.8 | 18.5 |
| 37A | 93 | 18 | 52 | 8 | 7.4 | 11.6 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 33A | 184 | 22 | 122 | 5 | 30.5 | 36.8 |
| 42C | 90 | 20 | 44 | 3 | 22.0 | 30.0 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 28A | 181 | 90 | 83 | 11 | 8.3 | 16.5 |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 32B | 138 | 34 | 86 | 8 | 12.3 | 17.3 |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 32A | 153 | 19 | 122 | 7 | 17.5 | 21.9 |
| Means.... | 125 | 24.7 | 77 | 10.6 | 9.3 | 15.3 | Means.... | 164 | 28.2 | 112 | 9.9 | 16.3 | 24.4 | Means.... | 232 | 56.2 | 143 | 9.7 | 24.7 | 53.2 |
| Standard deviation | 31.6 | 21.7 | 26.6 | 5.3 | 4.6 | 9.1 | Standard deviation | 66.7 | 18.7 | 71.7 | 8.7 | 10.1 | 25.9 | Standard deviation | 61.8 | 57.7 | 87.7 | 6.2 | 33.4 | 72.9 |

containing 5 cc. of fine sand, were autoclaved and stoppered with sterile corks. Each egg case was dipped in 70 per cent alcohol for 10 seconds, air dried, and placed in one of the prepared vials to which about 0.3 cc. of sterile distilled water had been added. Autoclaving the containers and dipping the capsules in alcohol were necessary to retard bacterial and mold growths, which otherwise developed rapidly on untreated capsules kept in the humid atmospheres of nonsterile containers. It appeared that certain of the molds destroyed eggs, particularly if mycelia penetrated the capsule and grew into the protoplasm of the eggs. Vials were corked until hatching occurred.

## RESULTS AND DISCUSSIONS

### I. PARTHENOGENESIS

Rau (1940) did not report parthenogenesis of the American roach; Gould and Deay (1938), and Nigam (1933) stated that they obtained negative results. During the course of the present experiment, 25 virgin females were isolated and observed. Altogether, they produced over 200 oothecae. Only 2 of the females produced no eggs, while 5 formed but 1 capsule each during the entire observation period. The egg cases from these unmated females were dropped at irregular intervals and were often imperfect. All the more nearly normal cases were retained and tested for viability. Nine of these capsules, from 3 different females, contained eggs from which apparently normal nymphs emerged. As many as 13 nymphs hatched from 1 capsule. An attempt to rear some of these nymphs was made, but only 4 reached maturity. They were all females. The 25 unmated females which produced viable eggs had been isolated in stoppered pressure bottles since the day they molted to the adult stage. None had ever had access to a male. One female, number 20B, which produced 2 cases with viable eggs out of a total of 14 capsules, had been isolated since her ninth instar. Number 45A established the highest individual record for virgin females. She dropped 23 capsules during her adult life. Of these, 4 were viable. In view of these facts it seems that parthenogenesis is a possible but infrequent phenomenon for the American roach.

### II. DIFFERENCES BETWEEN SERIES A AND SERIES B

Table 1 presents individual oviposition and longevity records for Series A, which included Group I (male present throughout adult life of female); Group II (male present only during preoviposition period); and Group III (females with delayed mating). These roaches were caged individually or as pairs in 1,000-cc. pressure bottles. Table 2 contains similar information for Series B from Group I (male present throughout adult life of female); Group II (male present during only preoviposition period); and Group IV (virgin females). These roaches of Series B were confined in 250-cc. flasks. Means and standard deviations are listed for all the results. The "Preoviposition Period" in the column headings is the time from adult emergence to formation of the first egg case. "Productive Period" is the number of days between appearance of first and last egg cases. "Days between Egg Cases" were determined by subtracting 1 from the number of egg cases and then dividing the number of productive days for each female by this remainder. "Days per Egg Case" were derived by dividing the adult life-span by the number of egg capsules. While there is a close relationship between the sets of figures in the last two columns mentioned, the number of days per egg case presents a clearer picture of the time required to produce 1 egg capsule in each of the groups of females.

For convenience of comparison and discussion, data from Tables 1 and 2 are summarized in Table 3.

Table 3 readily demonstrates the trends and differences which will be discussed in subsequent paragraphs. In both Groups I and II the females in the larger containers (1) lived longer, (2) began oviposition earlier, (3) produced eggs over a longer period, (4) produced more egg cases, and (5) dropped egg capsules more frequently. When these results were analyzed statistically, the differences between the means of Series A and Series B were found in every instance to be highly significant.[2]

The difference in the results from the two series appears to lie primarily in the shortened adulthoods of the roaches which were caged in the small containers. This decreased longevity is reflected in the shortened production periods and in the decreased number of capsules for females from Series B. The adult life-spans were probably decreased because of the restricted surroundings under which the females of Series B were forced to exist.

TABLE 3

SUMMARY OF DATA FROM SERIES A AND SERIES B

| Series | Group | Adulthood (Days) | | Preoviposition Period (Days) | | Production Period (Days) | | Number of Egg Cases | | Days between Egg Cases | | Days per Egg Case | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Av. | Range | Av. | Range | Av. | Range | Av. | Range | Av. | Range | Av. | Range |
| A.... | I | 223 | 120–398 | 15.6 | 9–36 | 183 | 63–333 | 28.9 | 10–52 | 6.5 | 5–9 | 8.4 | 6–18 |
| | II | 267 | 93–481 | 17.0 | 6–24 | 216 | 63–446 | 22.8 | 8–46 | 10.7 | 6–22 | 13.6 | 7–32 |
| | III* | 410 | 247–706 | 47.8 | 22–89 | 117 | 1–296 | 4.4 | 0–17 | 27.3 | 18–43 | 86.7 | 20–208 |
| B.... | I | 125 | 90–214 | 24.7 | 8–100 | 77 | 30–112 | 10.6 | 3–21 | 9.3 | 5–22 | 15.3 | 6–31 |
| | II | 164 | 91–268 | 28.2 | 13–73 | 112 | 27–249 | 9.9 | 3–26 | 16.3 | 7–35 | 24.4 | 10–65 |
| | IV | 232 | 138–356 | 56.2 | 14–188 | 143 | 1–288 | 9.7 | 1–23 | 24.7 | 8–144 | 53.2 | 9–273 |

*Oviposition data for this group only for period *prior* to introduction of male. Additional data for this group in Table 5.

It was difficult to maintain clean conditions in the small 250-cc. containers. This fact and the limited space in which the roach could exercise were probably the primary causes for the decreased longevity of the Series B roaches. Probably these factors also explain the increased interval between egg cases. However, in spite of the differences, the two series show identically the same variations between groups; and the means for Groups I and II of Series A vary in a direct and orderly fashion with those of similar groups in Series B.

The variability within the different groups shows a very definite trend. There was much less variability among the roaches which were caged in the large containers. The least variability was registered by Group I in Series A. These females had males present throughout their adulthood and were caged in large 1,000-cc. containers. Thus, the environment was entirely favorable to the maintainance of laboratory specimens. The variability increased either with a decrease in the size of the container or with a decrease in the amount of time that a male was present. Thus Groups III and IV were more variable than Group II; Group II was more variable than Group I; and Series B, as a whole, was more variable than Series A.

[2] The authors are indebted to Dr. George W. Snedecor, director of statistical laboratory, Iowa State College, for suggestions regarding statistical treatment of the data of this paper.

With the exception of those from Group III, all the data in Tables 1 and 2 were analyzed by a *t*-test method as outlined by Snedecor (1938). In addition, Series A and B were combined, and then Groups I and II were tested by a similar method. Table 4 gives a summary of the statistical analysis. The results are recorded as highly significant (H.S.), significant (S.), and not significant (N.S.). Although results which were not significant were often obtained, the means in both series show identically the same trends.

### III. PREOVIPOSITION PERIODS

Differences in preoviposition periods between Group I and Group II in the two series are not significant when compared in their own series. The normal preoviposition period appeared to be about 16.0 days. The increased preoviposition time for Series B may be attributed to the size of the container. The smallness of the flask made copulation ex-

TABLE 4

RESULTS FROM THE STATISTICAL ANALYSIS OF GROUPS I, II, AND IV

| DATA COMPARED | GROUPS COMPARED | | | | |
|---|---|---|---|---|---|
| | Series A | Series B | | | Series A and Series B |
| | Groups I and II | Groups I and II | Groups II and IV | Groups I and IV | Groups I and II |
| Longevity | N.S.* | N.S. | S. | H.S. | N.S. |
| Preoviposition period | N.S. | N.S. | N.S. | N.S. | N.S. |
| Production period | S. | N.S. | N.S. | N.S. | H.S. |
| Egg cases per female | N.S. | N.S. | N.S. | N.S. | N.S. |
| Days between egg cases | H.S. | S. | N.S. | N.S. | H.S. |
| Days per egg case | H.S. | N.S. | N.S. | N.S. | H.S. |

* N.S. = not significant; S. = significant; H.S. = highly significant.

tremely difficult, and undoubtedly delayed the genesis of oviposition. When the reproductive apparatus is mature, association with a male and, therefore, an opportunity for mating seemed to initiate an early beginning of egg production in a newly molted adult female. Both Group III and Group IV had preoviposition times which averaged about 50 days. Here the absence of a male delayed onset of ovarian activity, even though the mechanism for egg production was ready. The shortest preoviposition time was 6 days for number 10C of Group II, and the longest was 188 days for virgin 33B of Group IV. When newly molted adult females were placed with males, in large containers, there was an average of about a 16-day period before the production of the first egg case. Those confined in smaller flasks averaged about 26 days in their preoviposition times. Both of these figures are higher than the average of 13.4 days published by Gould and Deay (1938).

### IV. RATE OF OVIPOSITION

The effect upon egg production of temporary, permanent, or no association of females with males was manifested in various ways. Females in Group I, Series A, with males present throughout their entire adult life-span, dropped an egg case on an average of

every 6.5 days during their productive period; in Series B, the interval was 9.3 days. One female—No. 10B of Series A—produced an egg case every 5.0 days for 179 days. In contrast, when males were never present, virgin females produced capsules at very irregular intervals, averaging 27.3 days apart in Series A and 24.7 days in Series B. Females of Series A, paired with males only during the preoviposition period, produced egg cases on an average of every 10.7 days during their productive periods; the average for a similar group in Series B was 16.3 days. Consideration of these data makes it apparent that the presence of a male decreased the interval between egg cases. Similarly, the number of days per egg case is greater when males are absent and shows the same general trend (see Table 3). In other words, a female produces more eggs per day if a male is constantly present than if he is present only during the preoviposition period. Also, if the male is present only before egg production begins (a period of about 2 or 3 weeks), the rate of oviposition is greatly increased over that of the virgin female.

The number of egg capsules per female does not vary significantly within Series A or Series B. The fact that females in Series A dropped more cases than those in Series B was undoubtedly due to the increased adult life-span in the former series. A definite trend which may be of some importance was evident in both series; Group I produced more capsules than Group II, and Group II produced more than the virgin females. For the groups of Series A, the averages are, respectively, 28.9, 22.8, and 4.4. One female—No. 7B in Group I—with a male always present, produced a total of 52 capsules. Number 25B, in Group II, with a male only during her preoviposition period, dropped a total of 46 capsules during her productive period. After almost a year's absence from a male, she was still producing viable eggs. The largest number of oothecae produced by a virgin female was 23. In both series the tendency for females to produce imperfect eggs was decidedly more pronounced in Group II than in Group I. These imperfections were especially noticeable as the females became older.

### V. RELATIONSHIP BETWEEN LONGEVITY AND OVIPOSITION

Longevity of a mated female roach and the duration of her egg-producing ability are naturally closely allied, especially when male and female are together throughout the entire life-span of the female. As would be expected, high productivity generally occurred when the adult life-span was long. However, this is not always true for unmated females, for, in 2 instances, no egg cases were produced and in 5 cases not more than 1 capsule was formed, even though the period of adulthood was of longer average duration than that of mated females. Consequently, the productive period of such females was recorded at 1 day or less. The question arises as to why life-spans for unmated females of Group III should be greater than those of Group II and why this latter group should live longer than Group I. Groups I and II did not differ significantly (Table 4), but the *t*-value (1.911) was very close to the significant level (2.008), and the trends are identical in both series. Apparently this phenomenon can also be connected with greater egg production, which in turn is associated with presence of a male. Although females in Group II had longer production periods and longer imaginal life-spans than those in Group I, they did not produce more egg cases per female. Females of Group I produced capsules at a faster rate and also died sooner. It appears that energy necessary for formation and development of eggs may actually have reduced the longevity of these roaches. Data for Group III would seem to substantiate the above hypothesis, since this group far outlived the other two and also produced many less capsules per female.

The rate of ovarian activity was apparently slowed as the roach became older. This was evidenced by the increased time intervals between capsules. These increased intervals were accompanied by imperfect capsule formation. As noted above, this was particularly true for Group II.

The oldest roach encountered in this entire series of experiments was No. 22A, a member of Group III's (Ser. B) virgin females, which lived 706 days and produced only 11 egg cases. The average longevity for the females of Group I in Series A was 223 days and is probably the most accurate longevity figure listed. This is only 51 per cent of that reported by Gould and Deay (1938), and 72 per cent of the figure given by Rau (1940). No adequate explanation for the differences can be offered at this time, although Gould and Deay must have used a highly selected sample of roaches for computing their average life-span. They record a minimum lifetime of 102 days. It has been the experience of the writers that, even under excellent rearing conditions, a considerable number of adults die before the hundredth day after the final molt. In fact, our records show that 22 per cent of newly molted individuals died before reaching the age of even 50 days.

Only a few records were kept on longevity of male roaches. Nine males with known molting dates were observed to live more than 90 days. Their adult life-spans varied between 91 and 362 days, with an average of 200 days. In the American cockroach, as in many other animal species, the female seems to have the characteristically longer life-span.

### VI. DELAYED MATING

Table 5 presents the oviposition record of the 8 females from Group III of Series A, which had not mated until males were introduced after varying lengths of time ranging from 96 to 389 days after the final molt. The males were then removed when the next egg case appeared. With two exceptions, all dropped egg cases within 20 days or less after introduction of the male, and in several instances they continued to produce capsules for a considerable period. For 5 females the days between egg capsules were remarkably few. Although they had not been producing capsules very rapidly, the ability was apparently latent, for eggs were produced when the reproductive organs were properly stimulated. Only No. 22A did not show an accelerated egg output. Numbers 22A, 16A, and 48A did not show as great an increase as did the other 5 females. This may have been due to their advanced age when the males were introduced. Number 22A, for example, already had an adult age of 389 days before being placed with a male. Eggs which were produced after removal of the male were often viable. None of these females had produced parthenogenetic offspring prior to introduction of male roaches.

Data in Table 5 present in another form the effect of presence of males upon production of eggs by females. The reason for this effect has been only partially ascertained. Mating is of primary importance. Some roaches of Group III, Series A, had lived from a third to three-fourths of their adult lives before they had an opportunity to copulate, but after mating they produced capsules containing fertile eggs for from 2 to 4 months after introduction of the male. In two cases (Nos. 28B and 46A), the adult females had lived, respectively, 184 and 98 days without producing even 1 egg case. After the male was added, they formed 17 and 12 capsules, respectively, in their remaining lifetimes of 125 and 223 days. Twelve nymphs emerged from one of the oothecae dropped by No. 29A, which had already had an adult virgin life of 336 days before mating. Examination of other data in Table 5 will show further evidence of the effect of the male's presence in

increasing the average number of capsules per female, in decreasing average number of days between egg cases, and in decreasing the average number of days per egg case.

### VII. STORAGE OF SPERM

Although copulation was actually observed in only one instance, it is assumed that mating took place during the preoviposition period. The act of copulation may be stimulatory in itself, but it seems improbable that this stimulation would be carried over through the long period of time after the male had been removed from the female, as with females in Group II, Series A. If the act of copulation is to be considered as the determining factor, this carry-over would be necessary, in view of the prolonged period of egg production in Group II, in which males were removed as soon as the first egg case appeared. However, it may be that copulation induces some physiological change in the female which causes oviposition to begin and to be continued.

TABLE 5

OVIPOSITION RECORDS OF GROUP III, SERIES A, BEFORE AND AFTER INTRODUCTION OF MALE

| Roach Number | Without Male | | | | | After Male Added | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Days since Last Molt | Preoviposition Period (Days) | Number of Egg Cases | Days between Egg Cases | Days per Egg Case | Days until Death | Days until Next Egg Case | Number of Egg Cases | Days between Egg Cases | Days per Egg Case |
| 16A | 389 | 56 | 7 | 43.4 | 55.6 | 203 | 55 | 8 | 16.8 | 25.4 |
| 22A | 343 | 26 | 8 | 20.3 | 42.9 | 363 | 13 | 3 | 48.0 | 121.0 |
| 29A | 336 | 29 | 17 | 18.2 | 19.8 | 100 | 15 | 9 | 8.6 | 11.1 |
| 27B | 208 | 22 | 1 | ..... | 208.0 | 95 | 4 | 14 | 6.4 | 6.8 |
| 28B | 184 | ..... | 0 | ..... | ..... | 125 | 40 | 17 | 4.3 | 7.4 |
| 46A | 98 | ..... | 0 | ..... | ..... | 223 | 19 | 12 | 8.6 | 18.6 |
| 47A | 98 | 89 | 1 | ..... | 98.0 | 269 | 11 | 17 | 6.1 | 15.8 |
| 48A | 96 | 65 | 1 | ..... | 96.0 | 151 | 18 | 3 | 40.0 | 50.3 |
| Means | ..... | ..... | 4.4 | 27.3 | 86.7 | ..... | ..... | 10.4 | 17.4 | 32.1 |

Spermatozoa evidently can be stored by the female for long periods of time. Number 25B of Group II, Series A, presents a typical example. She produced a case containing fertile eggs 346 days after her first capsule appeared. For approximately 8 months following removal of the male, her egg cases hatched out 13–15 nymphs per capsule. Throughout the remaining year of her productive period only 6 or 7 nymphs emerged from each case. It appears that sperm cells may have been stored throughout this entire period, but because of their decreasing numbers fewer fertilized eggs were produced during the latter part of the productive period. The number of days between egg cases was gradually increased during the latter part of her life. For all of Group II it can be stated generally that the number of viable eggs per case decreased and the number of days between capsules increased as the roaches grew older. In Group I, in which males were always present, the percentage of viable eggs did not decrease with advancing age, even though fewer capsules may have been formed at longer intervals. In addition to de-

creased viability, many later capsules from Group II were markedly imperfect. It is possible that actual presence of either spermatozoa or of some substance associated with sperm may serve as an agent which stimulates the production of eggs and egg capsules. Stimulation from stored sperm would satisfactorily explain the maximum reproductive activity in Group I, where mating could occur freely; the decrease in egg production in Group II, in which sperm from perhaps only one mating was being slowly used up; and the small number of capsules in Group III, where no possibility of mating was allowed. It would also explain why the females in Group II produced fewer and less perfect egg cases as they became older. The time interval between egg cases increased for females of Group I as well as for those in Group II as they aged; but there were fewer imperfect capsules, and the slowing-down in rate of capsule production was less in Group I. Possibly both copulation and presence of stored spermatozoa are responsible for the observed differences.

Data listed in Table 6 offer further evidence in support of the suggestion that stored spermatozoa have an effect upon egg production. These females of Group V had been

TABLE 6*

OVIPOSITION RECORDS OF GROUP V OF SERIES B

| Roach Number | Adulthood (Days)† | Production Period (Days)† | Number of Egg Cases | Days between Egg Cases | Roach Number | Adulthood (Days)† | Production Period (Days)† | Number of Egg Cases | Days between Egg Cases |
|---|---|---|---|---|---|---|---|---|---|
| 19A..... | 240+ | 211+ | 28 | 7.5 | 18A..... | 204+ | 184+ | 24 | 8.0 |
| 13A..... | 237+ | 221+ | 31 | 7.4 | 11A..... | 188+ | 167+ | 23 | 7.6 |
| 14A..... | 235+ | 223+ | 33 | 6.8 | | | | | |
| 15A..... | 209+ | 196+ | 17 | 12.3 | Means..... | 219+ | 200+ | 26.0 | 8.0 |

* Females with males during the first four weeks of adult life.

† Exact periods for adulthood and egg production cannot be given here, since these specimens were taken from stock cultures after molting. See description of groups under "Material and Methods."

with many males in a large stock culture cage for several weeks prior to their isolation in the smaller flasks of Series B. The average number of days between egg cases from females of this group was less than half the average for those females of Group II in Series B, which had been kept in the same type of container but which had the possibility of mating only during the preoviposition period. This was true in spite of the fact that Group V females had longer productive periods and thus had time to produce more eggs. Perhaps these females had received more spermatozoa because of more frequent copulations. The various aspects of the oviposition records for this group of females are as good as those of Group I in Series B and are definitely better than those of Group II of the same series. Prior to isolation, Group V females had adequate opportunity for promiscuous mating in the stock cages. Thus copulation took place in freer surroundings, and, since males were not placed in the individual containers, the females were never subjected to as cramped quarters as were those of Groups I and II of Series B. Therefore, the size of the container may have been one of the factors which contributed to the above-mentioned differences within the same series. However, females of Group V did not produce so many eggs or produce them so rapidly as did those of Group I in Series A. Here the lack of agreement must be explained by the difference in the length of time of

association with male roaches. Both of the factors of cramped living conditions and of opportunity for mating help to explain the differences between the records of Group V and of Series A's Group II. Close confinement decreased the longevity of Group V, but the prolonged opportunity for copulation and storage of sperm during the early period of adult life apparently resulted in an acceleration of egg production.

### VIII. HATCHING TIMES

Some records of hatching times were obtained, and results are recorded in Table 7. This table presents data from oothecae, produced by members of Groups I and II, at room temperature and at 29° C. Hatching records for eggs at room temperature (17°–28° C.) were gathered during winter months when the laboratory was cool at night and undoubtedly present a longer incubation period than would be found in warmer summer months. An egg case was usually carried by the female for 2 days before it was dropped. Haber (1920) found that his specimens generally deposited their capsules "one day after appearance," although in at least 1 case, the ootheca was not deposited until "six days after its appearance." In experiments here described, as many as 18 nymphs emerged from 1 capsule, but this occurred on only one occasion. There was an average of 12 eggs per case. The cases which Lefroy (1909) examined averaged 16 eggs per capsule. Bugnion's (1923) specimens averaged 14 eggs per case; Adair's (1923), about 21.5.

TABLE 7

INCUBATION RECORDS FOR EGG CASES

| Group | Number of Cases | Number of Days To Hatch | | Days Carried by Female | | Number of Nymphs Hatched | | Average Number Eggs Not Hatched per Case |
|---|---|---|---|---|---|---|---|---|
| | | Av. | Range | Av. | Range* | Av. | Range | |
| | Room Temperature (17°–28° C.) | | | | | | | |
| I | 61 | 53.1 | 35–72 | 2.3 | 1–5 | 12.9 | 5–16 | 0.3 |
| II | 20 | 51.4 | 36–64 | 2.2 | 1–5 | 13.0 | 7–15 | 1.0 |
| Means | ..... | 52.7 | ..... | 2.3 | ..... | 12.9 | ..... | 0.5 |
| | Constant (29° C.) | | | | | | | |
| I | 15 | 39.1 | 36–43 | 1.5 | 1–5 | 11.5 | 2–18 | 0.7 |
| II | 25 | 40.7 | 38–48 | 2.0 | 1–8 | 10.1 | 2–15 | 1.6 |
| Means | ..... | 40.1 | ..... | 1.8 | ..... | 10.4 | ..... | 1.0 |

* Cases were sometimes carried less than 24 hours, but exact hourly time was not determined and is here recorded as 1 day.

### IX. MISCELLANEOUS OBSERVATIONS

Many females ate their own egg capsules, even though adequate food was always present. At one time or another each of them consumed at least 1 egg case. However, a few females regularly ate the capsules as rapidly as they dropped them. Seemingly, they

developed a preference for the eggs and ate them even in the presence of sufficient available food of a type which has seemed adequate during several years of laboratory rearing of this roach.

Several females attacked and killed males when the latter were present. One female was especially vicious and attacked each new male as he was introduced into the container. Most of such males had molted less than 2 days previously. Older males were more capable of defending themselves against attacks of these cannibalistic females. No similar cases in which males attacked females were observed.

### X. COMPARISON OF RESULTS

Table 8 presents some comparable results of various explorations into the biology of the American roach. Discrepancies in published reports seem to indicate that perhaps

TABLE 8

COMPARISON OF RECORDS OF BIOLOGY OF THE AMERICAN ROACH

| Source | Adulthood in Days | | | | Preoviposition Period (Days) | Days between Egg Cases | Egg Cases per Female | Days of Incubation | Parthenogenesis Reported |
|---|---|---|---|---|---|---|---|---|---|
| | Female | | Male | | | | | | |
| | Av. | Max. | Av. | Max. | | | | | |
| Gould and Deay | 438 | 588 | ..... | ..... | 13.4 | 5.9 | 58.8 | 55.3 | − |
| Griffiths and Tauber | 225 | 706 | 200 | 362 | 20.0 | 6.8 | 21.1 | 53.0 | + |
| von Fischer | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 42.0 | − |
| Nigam | ..... | ..... | ..... | ..... | ..... | 4–10 | ..... | 28.0 | − |
| Rau | 310 | 330 | 82 | 175 | ..... | 7.4 | 11.9 | 43.0 | − |
| Sein | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 38.0 | − |

"strains" of the American roach have built up in certain localities. With an insect as adaptable as the cockroach, it would not be surprising to find slight biological differences recorded for populations in localities ecologically separated as widely as Egypt and central North America. Averages taken from this paper are derived from both Group I's, with the exceptions of those for longevity and incubation times.

### SUMMARY

1. Parthenogenesis was found to occur in 9 egg capsules formed by 3 out of 25 unmated females of the American cockroach, *P. americana*. The phenomenon occurred in 4.5 per cent of the well-formed, seemingly normal capsules produced by virgin females. Unmated females produced many malformed egg cases, which were not retained and were not tested for viability.

2. Eighty-five females were divided into five groups: Group I was placed with males throughout the adult life of the females; Group II had males present only during the preoviposition period; Group III was composed of virgin females which were separated from males until the latter part of their lives; Group IV was made up of virgin females separated from males throughout their entire adult lifetimes; and Group V was with males during about the first 4 weeks of adult life of the females. Oviposition records

demonstrate that presence of a male, with the opportunity of mating, increases the number of egg capsules and also the rate of their production. Females of Group I produced an average of 20.1 capsules, which appeared successively at an average of 7.7 days apart; those of Group II, an average of 16.7 capsules 13.4 days apart; those of Group III, only 4.4 egg cases at intervals of 27.3 days; females of Group IV, 9.7 capsules every 24.7 days; and females of Group V, an average of 26 capsules which appeared every 8.0 days (the averages for Group III include only the data prior to the introduction of males).

3. It is suggested that copulation and the presence of spermatozoa or some substance associated with sperm cells, when stored in the female, are largely responsible for the genesis and prolonged continuance of oviposition.

4. The average preoviposition period for females in the large containers with access to males was 16 days, and the range varied from 6 to 36 days. For females with access to males, in the 250-cc. containers, the preoviposition periods average about 20 days, with a range of from 8 to 100 days.

5. Increased egg production is correlated with decreased longevity. Females in Group I lived an average of 181 days; those from Group II lived 218 days; and virgin females lived 295 days.

6. The average adult life-span for this selected group of roaches was 225 days for the females and 200 days for the males. Female adulthood varied from 90 to 706 days and that of the male from 91 to 362 days.

7. A maximum of 18 nymphs was found to emerge from 1 egg capsule, but the average number from 121 cases was 12. The incubation period over moist sand was 53 days at room temperature, which fluctuated between 17° and 28° C.; and 40 days at 29° C.

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# ACCUMULATION OF MANGANESE AND THE SEXUAL CYCLE IN OSTREA VIRGINICA

(One figure)

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SINCE the finding of manganese in blood, urine, teeth, gallstones, milk, and many internal organs of man (Bertrand et Medigreceanu, 1912*a*, 1912*b*, 1912*c*) the question whether its presence in animal tissues is accidental or whether this metal is an indispensable constituent of the normal organism has been debated in the scientific literature. Inasmuch as a comprehensive review of the subject has been made by Browning (1937), it will suffice to mention here only a few papers which deal with the role of Mn in the reproductive cycle and growth of animals. Kemmerer, Elvehjem, and Hart (1931) found that the addition of Mn to the food of mice increases the rate of their growth, and they attributed this effect to the stimulation of ovarian and testicular functions. According to Orent and McCollum (1931), Mn is essential in maintaining the normal development of embryonic and young tissues and in the production of a hormone, probably from the anterior lobe of the hypophysis, which is essential for the functioning of the testes and for the proper development and functioning of the mammary tissue. Of special interest are their observations that an Mn-free diet results in the degeneration of testes in the male rats, which otherwise develop normally, and leads to the loss of the maternal instincts in the females.

Recent investigations of Ehrishmann (1939) made on twenty generations of white mice showed that the animals fed on a balanced diet, including Mn in the form of brownstone ($MnO_2$), maintained weights above the controls. The total number of births and the proportion of sterile females were the same in both groups, but the number of stillbirths was significantly higher in the Mn-fed animals than in the controls.

Mn was found to be present also in the yolk of hen's eggs, where its content increases during the maturation of the ovaries (Bertrand and Medigreceanu, 1913). Undoubtedly, Mn is a regular constituent of plant and animal tissues and apparently has important functions in both. Fearon (1933) lists it as one of the "invariable microconstituents" which are probably present in all forms of life. In mammals it is mainly stored in the liver and in the kidneys (von Oettingen, 1935).

Scientific opinion regarding its physiology seems to incline at present to the conception of this metal as a coenzyme or a catalyst which stimulates the enzymes controlling carbohydrate metabolism and activates a number of vital reactions, such, for instance, as the oxygenation of monosaccharides, oxidation of cystine to cysteine, antitoxin formation, and respiration of animal tissues (Browning, 1937).

That Mn is needed for the propagation of diatoms has been shown by Harvey (1939). He found that a concentration of one part per thousand million is sufficient for vigorous growth of these algae and that the fertility of the natural sea water can, in some cases, be restored by adding 1–2 mg. of Mn per cubic meter.

[1] Published by permission of the director, U.S. Fish and Wildlife Service.

The occurrence of Mn in fresh-water lamellibranchs was demonstrated by Bradley (1907, 1910), who found that the tissues of *Anodonta* and *Unio* contain large quantities of this metal, varying from 0.293 per cent of dry weight in the muscles to 2.457 per cent in the mantle. Bradley found no significant seasonal variation in the Mn content in these species. Heuverswyn (1930), and Dubuisson and Van Heuverswyn (1930) demonstrated that in *Anodonta* the Mn is stored principally in the gills. Among marine mollusks Mn was found in the blood protein of *Pinna squamosa* (Griffith, 1892) and in the tissues of *Modiola modiolus* and *Pecten* (Bradley, 1910). Only traces of this metal were found by Bradley in *Mytilus edulis*. Bertrand and Medigreceanu (1913) report the presence of Mn in *Ostrea edulis*, *O. angulata*, *Pecten jacobaeus*, *M. edulis*, *Cardium edule*, and *Tapes decussatus*. The amount of Mn in these organisms, expressed by the authors in milligrams per 100 gm. of fresh tissues, varies from 0.10 mg. in *O. edulis* to 3.664 mg. in *P. jacobaeus*.

In the present investigation samples of oysters were collected at regular intervals from the experimental oyster bed in Long Island Sound at Charles Island, Connecticut. All the oysters used in this work were three years old in the spring of 1932, when they were obtained from a private oyster farm in the vicinity of New Haven and planted on the experimental area under observation. Selected for their uniform shape, size, and general good quality, they represented as homogeneous a population as it was possible to obtain in Long Island Sound.

The period of observations extended from May 7, 1932, until April 3, 1935. Each sample, comprising ten oysters taken at random, was delivered to the laboratory for analysis. The author is greatly indebted to Dr. Victor L. Loosanoff, director of the U.S. Fishery Biological Laboratory at Milford, Connecticut, for co-operation in obtaining the samples and preserving them for chemical analysis. Analytical work was carried out at the Woods Hole and Washington laboratories with the assistance of Charles D. Weber, temporary assistant of the U.S. Bureau of Fisheries.

## METHOD

Within 2 or 3 hours after being taken from the water the oysters were opened, and their bodies separated from the shells with a sharp stainless-steel knife and placed for 15 minutes on filter paper to remove the superfluous water. Then they were transferred into a porcelain dish, kept in an oven for 24 hours at 60° C., and dried to constant weight at 100°. Dry samples were pulverized in a glass mortar, mixed, and stored in glass bottles. For ashing, 1 to 2 gm. of the dried material was placed in a silica crucible and charred over an electric hot plate. Then it was put in the muffle furnace and ashed just below red heat for about 2 hours. Upon cooling, 1 ml. of concentrated $HNO_3$ was added, and the sample evaporated to dryness on an electric hot plate. The temperature during evaporation was kept below the boiling-point. Then the sample was placed in the muffle again and kept just below red heat for 1 hour longer. Finally it was dissolved in 10 ml. of HCl (1:1) and diluted to 100 ml.

For Mn determination Richards' procedure, fully described in his paper (1930), was followed. A comparison of color was made with a series of standards containing 0.05, 0.03, 0.02, and 0.01 mg. of Mn in 50 ml. The results are expressed in milligrams of Mn per kilo of dry weight.

The reliability of chemical determinations can be evaluated from the analyses of ten duplicate samples shown in Table 1. In the majority of these samples the difference be-

tween the two analyses was less than 5 per cent, exceeding this figure in only two cases. Since the seasonal fluctuations in the Mn content described below far exceed the experimental error, the results are considered satisfactory.

## OBSERVATIONAL DATA

The manganese content of the oysters follows a definite seasonal cycle, reaching its lowest level, from 7 to 11 mg. per kilo of dry weight, in winter and attaining a maximum from 28 to 57 mg. in summer. This trend of the manganese curve is clearly shown in Figure 1, which represents the plotting of the data obtained during a period of observations covering almost three years. These changes cannot be attributed to length of life or racial differences, for the oysters used in the analysis were of the same age at the beginning of the observations and represented samples of a homogeneous population. It may be suggested that the relatively high Mn content in summer and its low

TABLE 1

DUPLICATE ANALYSES OF OYSTER TISSUES FOR MN

| Sample Nos. | Mn per Kilo of Dry Weight | | Per Cent Difference |
|---|---|---|---|
| | Test 1 | Test 2 | |
| 159–159′ | 10.02 | 9.95 | 0.7 |
| 167–167′ | 8.04 | 8.52 | 5.7 |
| 173–173′ | 8.08 | 8.21 | 1.58 |
| 178–178′ | 8.03 | 7.92 | 1.44 |
| 191–191′ | 14.8 | 14.7 | 0.07 |
| 192–192′ | 26.2 | 25.8 | 1.53 |
| 197–197′ | 18.4 | 16.9 | 8.15 |
| 1014–1014′ | 17.5 | 17.5 | 0 |
| 1019–1019′ | 8.85 | 8.67 | 2.26 |
| 1024–1024′ | 17.0 | 17.1 | 0.06 |

content in winter is purely accidental, arising from the presence of marine algae in the digestive track of oysters. At low temperatures, when the feeding mechanism ceases to function (Galtsoff, 1928), the stomach and intestines of the oysters are usually devoid of food. This fact was fully confirmed by the present investigations. Each time a sample of oysters was collected for analysis a number of them were dissected and their digestive tracts examined. In winter no food or feces were found in the intestines, and the crystalline style, which is always present when oysters are actively feeding, was absent. Conversely, during the feeding seasons the stomach and intestine always contained food. Starving the oysters by keeping them in filtered water for at least 24 hours before opening them for chemical analysis was impractical, for the samples were also used for the determination of glycogen and water content, which might have been affected by this treatment.

In spite of the known high Mn content of phytoplankton (Thompson and Wilson, 1935), the presence of algae in the intestinal tract cannot account for the observed seasonal fluctuations of this element in oysters. First, the amount of food in the intestinal tract constitutes but a very small portion of the total weight of an oyster meat; second, the Mn content rapidly decreases during the second half of August and Septem-

ber (Fig. 1), when oysters are actively feeding and accumulating glycogen. Furthermore, analysis of the visceral mass, which comprises the entire intestinal tract and digestive gland, shows that in summer this part of the body contains less Mn than is found in the gills and ovaries (Table 2).

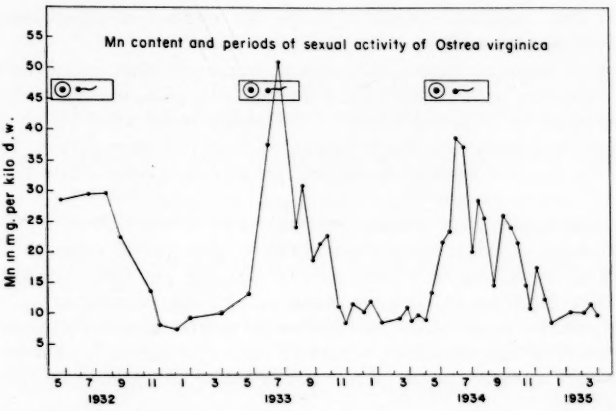


FIG. 1.—Seasonal changes in the manganese content of oysters in Long Island Sound. Inserts with outlines of the egg and sperm indicate approximate periods of ripening of the gonads and spawning of oysters.

TABLE 2

MANGANESE CONTENT OF OYSTER TISSUES
(In milligrams per kilo, dry weight)

| Date | Muscle | Gills | Mantle | Visceral Mass | Testis | Ovary |
|---|---|---|---|---|---|---|
| June 20, 1934 | ..... | ..... | ..... | ..... | ..... | 56.8 |
| July 3, 1934 | 5.22 | 35.1 | ..... | 18.4 | ..... | 59.6 |
| July 3, 1934 | ..... | ..... | ..... | ..... | ..... | 51.0 |
| July 6, 1934 | 4.37 | 38.6 | ..... | 8.88 | 4.63 | ..... |
| July 18, 1934 | ..... | ..... | ..... | ..... | 7.25 | ..... |
| Sept. 5, 1933 | 4.94 | 23.1 | 17.0 | 17.5 | ..... | ..... |
| Sept. 8, 1933 | 3.61 | 25.5 | 14.2 | 8.85 | ..... | ..... |
| Jan. 12, 1932 | 9.27 | 17.1 | ..... | ..... | ..... | ..... |
| Jan. 3, 1932 | 4.13 | 18.0 | 8.71 | ..... | ..... | ..... |

According to the author's determinations, the water content in the tissues of oysters from the experimental bed at Charles Island varied during the period of observations from 74.3 to 86.6 per cent. Since manganese is present in the sea water, it may be suggested that its fluctuations in the oysters are due to the seasonal variations of this metal in the sea. Thompson and Wilson (1935) have found that in the Northeast Pacific the content of Mn in sea water varied from $0.2\times10^{-4}$ to $1.8\times10^{-4}$ mg. atoms per kilo of

water and that in the six samples collected at intervals throughout a year in San Juan Channel, Friday Harbor, the Mn fluctuated between $0.5\times10^{-4}$ and $1.5\times10^{-4}$ mg. per kilo. This concentration is so low that the contribution of Mn by the sea water present in the samples of oyster meats would amount only to a small fraction of a milligram of metal per kilo of dry tissues and therefore cannot influence our results. It is unreasonable to expect that seasonal variations in the Mn content of the Atlantic water are significantly different from those of the Pacific.

For analysis of separate tissues and organs, composite samples of ten oysters were prepared by removing with sharp scissors the mantle, gills, muscle, and gonads. The remains, designated as "visceral masses," contained small amounts of gonad tissues which could not have been completely separated from the parenchyme. In the fall and winter, when the gonads were undeveloped, no attempts were made to separate the sexes.

Among various organs, ripe ovaries were found to be the richest in Mn, containing from 51.0 to 59.6 mg. of this metal per kilo of dry weight. On the other hand, testes were very poor in Mn, containing only from 4.63 to 7.25 mg. per kilo. The gills had rather high content, varying from 17.1 mg. in winter to 38.6 mg. in summer.

Analyses of mantle made in the fall just after spawning and in January showed a fluctuation from 8.71 mg. per kilo in winter to 14.2–17.0 mg. in September. In the adductor muscle only a small amount of Mn can be found throughout the year (from 3.61 to 9.27 mg. per kilo).

The large difference in the Mn content of testes and ovaries is probably due to the much greater proportion of nuclear material in the sperm and the predominance of cytoplasm in the eggs. This may suggest that the Mn is accumulated primarily by the cytoplasm.

Seasonal fluctuations of the total Mn content in the random samples (Fig. 1) show that the peaks of the manganese curve coincide with the periods of maximum gonad development and sexual activity of the oyster. The latter are approximately indicated in the diagram by inserts showing outlines of the egg and sperm. One must bear in mind that gonad development is a continuous process which begins late in fall or early in winter after a complete reabsorption of the unspawned eggs or sperm and proceeds throughout the winter. The onset of warm weather in May and early in June accelerates the propagation of sex cells, and full gonad ripeness is reached about the middle of the summer. Upon shedding the greater part of the eggs and sperm, the body of the oyster shrinks, the sex cells which may have remained in the follicles are rapidly absorbed by the invading phagocytes, and the sex gland enters into an indifferent phase, when the sex of the mollusk cannot be recognized. This after-spawning period is accompanied by a rapid loss of manganese. A low Mn level is continued throughout the hibernation period, when oysters cease feeding.

It appears obvious that the observed seasonal fluctuations in the concentration of manganese in samples of the oyster population of Long Island Sound are primarily due to the accumulation of this metal by the ovaries. Gills seem to be the other organ which stores this metal in quantities greater than those found in the other parts of the body. It is of interest to note that in summer the gill of the females, as well as of the males, have a rather high Mn content (35.1 and 38.6 mg. per kilo of dry weight, respectively) and that in winter the Mn content of this organ decreases to about one-half of its summer level. These observations, although incomplete, suggest that seasonal fluctuations

in Mn are not confined to the ovaries but occur to a smaller extent in the gills of both sexes.

The significance of Mn in the physiology of the oyster is not clear. It is possible that as a catalyst this metal plays a role in the storage and utilization of glycogen by the oysters. Its effect on the development of the gonads and on sex change in the oyster is now being studied experimentally.

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# STUDIES OF THE PRETRAUMA FACTORS NECESSARY FOR PLACENTOMATA FORMATION IN THE RAT[1]

(One figure)

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IN A previous study (Rothchild, Meyer, and Spielman, 1940) we reported the effect of progesterone alone or in combination with estradiol on the formation of placentomata in rats castrated on the fourth day of pseudopregnancy. The control of placentomata size and histology was shown to depend upon the progesterone dose and the amount of estradiol administered concomitantly. It was shown also that, while placentomata of normal size and histology could be formed with progesterone alone, the minimum effective amount of the corpus luteum hormone could be decreased by the addition of small amounts of estradiol.

The question, however, of the pretrauma physiology of the uterus in placentomata formation remained for the most part unanswered. We visualized at least three controlling factors—estrogen, progesterone, and time—as acting during the pretrauma period and attempted to set up experimental variations of these factors specifically designed to answer certain questions. These were: (1) Does the degree of estrogen stimulation of the uterus before progesterone treatment affect the size of placentomata formed when a constant amount of progesterone is administered? (2) Is estrogen stimulation of the uterus previous to progesterone stimulation an essential part of the physiology of placentomata formation? (3) Under what experimental conditions most nearly simulating the natural ones is it possible to maintain the sensitivity of the uterus to trauma for indefinite periods of time, and under what experimental conditions can this sensitivity be destroyed?

Four experimental procedures were used in answering these questions. Experiments I and II were designed to answer Questions 1 and 2, while Experiments III and IV were planned to answer Question 3. The procedures used and the results obtained are described below under separate headings for each experiment.

The progesterone used in this study was a noncrystalline preparation made from cholesterol by the Spielman and Meyer (1939) process and contained 10–50 per cent progesterone by weight. All lots were assayed on rabbits by the Corner and Allen (1929) method, and the doses used are expressed in Corner-Allen Rabbit Units (RbU). In several instances the results obtained with this preparation were compared with equivalent doses of crystalline progesterone,[3] and in all cases no significant differences were noted. The estrogen used was crystalline estradiol. All injections of progesterone

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[3] We wish to thank Dr. E. Schwenk of the Schering Corporation, Bloomfield, N.J., for the crystalline progesterone and estradiol used in these experiments.

were made in a volume of 0.10 cc. corn oil per injection and two injections were made daily. When estradiol was given concomitantly with progesterone it was added directly to the solution containing the progesterone without changing the volume of oil. Traumatization of the uterus was done by inserting a long needle into the lumen and piercing the antimesometrial wall in several places along its length (Rothchild, Meyer, and Spielman, 1940). In all cases this operation took place midway in time between the morning and evening injections on the designated day.

Several terms used below may bear defining. "Pretrauma" refers to the period of time in placentomata formation between the beginning of progesterone action and the actual traumatization of the uterus. "Posttrauma" refers to the time from traumatization of the uterus to the time of autopsy but may be interpreted broadly as the time of actual growth and degeneration of decidual tissue. "Estrogen pretreatment," "pretreatment dose," or amount of "estrogen pretreatment" may all be interpreted as any estrogen effect on a quiescent uterus just prior to the beginning of progesterone treatment. It should be clearly distinguished from the "pretrauma estrogen treatment" described in Experiment IV below, where the latter is additional treatment with estrogen during the time of the administration of progesterone.

## EXPERIMENTAL PROCEDURES

### EXPERIMENT I

*The amount of estrogen given before the injections of progesterone was varied while the total amount of progesterone, the total length of progesterone treatment, and the lengths of the pretrauma and posttrauma periods were kept constant.*

Fifty-six adult female rats, both virgin and parous, were castrated and allowed to go without treatment of any kind for a period ranging from 2 to 6 months. At the end of this time they were divided into seven experimental groups, each containing from 5 to 15 rats, and given the following treatment: Groups 1–6, inclusive, received a total of 0.05, 0.10, 0.22, 1.3, 6.7, and 33 γ of estradiol, respectively, in 4 injections of 0.05 cc. corn oil each for each animal during 24 hours. A minimum of 2 days was allowed to elapse between the time of the last injection and the beginning of the progesterone administration, to allow for the complete disappearance of cornified cells from the vaginal smear. The progesterone was administered to all rats in all the groups for a period of 8 days and the daily dose was 0.6 RbU. On the fourth day of progesterone administration the uterus was traumatized, and on the day after the last injection the animals were killed and autopsied. Group 7 consisted of 15 normal rats which were castrated in estrus, so that the effects of the estrogen secreted by the animals' ovaries could be compared with that of the injected estrogen. No time elapsed in this group between the castration and the beginning of progesterone treatment, since castrations were done late in estrus when the vaginal smears showed the presence of white cells. All other conditions were kept the same, however, as in the first six groups.

### RESULTS

The results are shown in Table 1. When the total pretreatment dose of estradiol was 0.05 γ, only 3 of the 8 rats treated reacted positively. Increase in the amount of estradiol administered resulted in an increase in the percentage of animals giving a positive reaction, without, however, resulting in any increase in the size of the placentomata.

(There was no statistically significant difference in the size of placentomata between the group of rats which received 33 γ of estradiol and that which received 0.05 γ of estradiol. There was also no significant difference between the latter group and that castrated in heat.)

EXPERIMENT II

*The pretrauma and posttrauma doses of progesterone were varied in amount, while the total length of progesterone administration and the lengths of the pretrauma and posttrauma periods were kept constant. Estrogen pretreatment was omitted.*

Thirty rats which had been castrated for 2–6 months without any further treatment were used. The total length of treatment with progesterone was 8 days, as in the case of Experiment I. Traumatization of the uterus was done on the fourth day and autopsy

TABLE 1

EFFECT OF VARIATION IN AMOUNT OF ESTRADIOL USED TO PRETREAT CASTRATE RATS ON THE FORMATION OF PLACENTOMATA

| Experimental Group | Total Dose Estradiol (γ) | Daily Dose Progesterone (RbU) | No. Rats | Placentomata | | Experimental Group | Total Dose Estradiol (γ) | Daily Dose Progesterone (RbU) | No. Rats | Placentomata | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | No. Rats with Placentomata | Size* (Mm.) | | | | | No. Rats with Placentomata | Size* (Mm.) |
| 1..... | 0.05 | 0.6 | 8 | 3 | 4.63 | 5..... | 6.70 | 0.6 | 6 | 6 | 4.08 |
| 2..... | 0.10 | 0.6 | 8 | 6 | 5.09 | 6..... | 33.0 | 0.6 | 6 | 6 | 4.55 |
| 3..... | 0.22 | 0.6 | 8 | 7 | 4.56 | 7†..... | ..... | 0.6 | 15 | 14 | 5.03 |
| 4..... | 1.30 | 0.6 | 5 | 5 | 4.34 | | | | | | |

* A total of 481 placentomata were measured, with an average number of 8.4 placentomata per rat and 60 placentomata per experimental group.

† Castrated in estrus, and 4 rats given crystalline progesterone.

on the day after the last injection. The rats were divided into four groups as follows: Group 1: Nine rats were given 0.6 RbU progesterone per day for 8 days; Group 2: thirteen rats were given 2 RbU or mgm. progesterone per day for 8 days; Group 3: four rats were given 2 RbU progesterone per day during the pretrauma period and 0.6 RbU progesterone per day during the posttrauma period; Group 4: four rats were given 0.6 RbU progesterone per day during the pretrauma period and 2 RbU progesterone per day during the posttrauma period.

RESULTS

The results are shown in Table 2. It can be seen that without estrogen pretreatment, 0.6 RbU progesterone per day was ineffective in producing placentomata. Positive results, however, were obtained when the pretrauma dose was 2 RbU of progesterone per day, irrespective of the posttrauma dose (Groups 2 and 3). (Seven of the 13 rats in Group 2 received crystalline progesterone.) This amount of progesterone, on the other hand, was not effective when it was given only during the posttrauma period, with 0.6 RbU as the pretrauma dose.

EXPERIMENT III

*The pretrauma period was varied in length, while the length of the posttrauma period, the daily amount of progesterone, and pretreatment with estrogen (castration in estrus) were kept constant.*

Sixty-nine adult virgin female rats were castrated late in estrus. Progesterone treatment was begun on the same day and continued daily for periods ranging from 6 to 24 days. The dose in all cases was 0.6 RbU or mgm. per day per rat. The rats were divided into eight groups according to the day on which the uterus was traumatized. These days were the second, fourth, sixth, eighth, tenth, twelfth, sixteenth, and twentieth days of progesterone administration. The posttrauma period was the same in all cases, namely, 5 days, the day on which the uterus was traumatized being considered as the last day of the pretrauma period and the first day of the posttrauma period. Four of the rats in the group traumatized on the fourth day and 4 of the group traumatized on the twentieth day received crystalline progesterone. (It will be noted that Group 7 of Experiment I and Group 2 of Experiment III are the same.)

TABLE 2

EFFECT OF VARIATION IN THE QUANTITY OF PROGESTERONE GIVEN DURING THE PRETRAUMA AND POSTTRAUMA PERIOD ON THE FORMATION OF PLACENTOMATA IN CASTRATE RATS NOT GIVEN ESTROGEN

| Experimental Group | Daily Dose Pretrauma Progesterone (RbU) | Daily Dose Posttrauma Progesterone (RbU) | No. Rats | Placentomata | | Experimental Group | Daily Dose Pretrauma Progesterone (RbU) | Daily Dose Posttrauma Progesterone (RbU) | No. Rats | Placentomata | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | No. with Placentomata | Size (Mm.) | | | | | No. with Placentomata | Size (Mm.) |
| 1..... | 0.6 | 0.6 | 9 | 0 | ..... | 3..... | 2.0 | 0.6 | 4 | 4 | 4.20 |
| 2..... | 2.0 | 2.0 | 13* | 11 | 4.15 | 4..... | 0.6 | 2.0 | 4 | 0 | ..... |

* Seven of these animals received crystalline progesterone.

RESULTS

Table 3 shows the pertinent results of this experiment. Placentomata formed in the majority of animals in each group with the exception of Group 1, in which the uterus was traumatized on the second day of progesterone treatment. There was a slight decrease in size of the placentomata with increase in length of the pretrauma period, but it is not statistically significant.

A histological examination of the placentomata formed in this and in Experiments I and II did not reveal anything unusual. Both the mesometrial and antimesometrial parts of the decidua were present, and in the proportions found in placentomata formed in normal intact pseudopregnant rats (Krehbiehl, 1937).

EXPERIMENT IV

*The lengths of the pretrauma and posttrauma periods, the daily amount of progesterone and the pretreatment with estrogen (castration in estrus) were kept constant. A variable fac-*

*tor was introduced by adding different amounts of estradiol to the progesterone during part of the pretrauma period and, in one group of rats, during the posttrauma period.*

Twenty-six adult female rats were castrated on the day of heat. Injections of progesterone were begun on the same day and continued for 12 days in all cases. The dose of progesterone used was 0.6 RbU per day per rat. The uterus was traumatized on the eighth day of progesterone treatment, and the animals were killed on the thirteenth day.

TABLE 3

EFFECT OF VARIATIONS IN THE LENGTH OF THE PRETRAUMA PERIOD ON THE FORMATION OF PLACENTOMATA WHEN ESTROGEN PRETREATMENT, DAILY PROGESTERONE DOSE, AND LENGTH OF THE POSTTRAUMA PERIOD WERE KEPT CONSTANT

| Experimental Group | Pretrauma Length (Days) | Number Rats | Number Positive | Placentomata Size* (Mm.) | Experimental Group | Pretrauma Length (Days) | Number Rats | Number Positive | Placentomata Size* (Mm.) |
|---|---|---|---|---|---|---|---|---|---|
| 1..... | 2 | 5 | 0 | ..... | 5..... | 10 | 7 | 7 | 4.64 |
| 2†..... | 4 | 15 | 14 | 5.03 | 6..... | 12 | 7 | 7 | 4.64 |
| 3..... | 6 | 8 | 7 | 4.58 | 7..... | 16 | 7 | 6 | 4.51 |
| 4..... | 8 | 7 | 7 | 4.93 | 8†..... | 20 | 13 | 11 | 4.18 |

* A total of 440 placentomata were measured, with an average number of 7.6 per rat and 63 per experimental group.
† Four rats given crystalline progesterone.

The rats were divided into the following groups. according to the manner of administration of estradiol (daily dose, number of days, and time of administration):

Group 1. 4 rats given 0.15 γ per day for 3.5 days *before* trauma
Group 2. 4 rats given 0.05 γ per day for 3.5 days *before* trauma
Group 3. 4 rats given 0.03 γ per day for 3.5 days *before* trauma
Group 4. 4 rats given 0.01 γ per day for 3.5 days *before* trauma
Group 5. 3 rats given 0.03 γ per day for 2.5 days *before* trauma
Group 6. 3 rats given 0.03 γ per day for 1.5 days *before* trauma
Group 7. 4 rats given 0.15 γ per day for 3.5 days *after* trauma

(See Fig. 1.) Vaginal smears were taken during the time of estradiol administration and for 1–2 days thereafter.

## RESULTS

The results are shown diagrammatically in Figure 1. Those rats which recieved 0.15 γ, 0.05 γ, and 0.03 γ of estradiol per day for 3.5 days before the traumatization of the uterus failed to form placentomata. Of these rats, those given 0.03 or 0.05 γ showed no evidence of being stimulated by estrogen as determined by the vaginal smear, whereas the rats which received 0.15 γ of estradiol showed marked vaginal cornification. Daily amounts smaller than 0.03 γ estradiol or this amount given for less than 3.5 days before trauma either did not prevent the formation of placentomata or prevented it only partially. (Three of the 4 rats which received 0.01 γ formed placentomata, all the rats which received 0.03 γ for 1.5 days formed placentomata, and 1 rat of 3 formed placentomata when the latter dose was given for 2.5 days.) That the effect of the estrogen is inhibitory only to the sensitizing action of progesterone is shown by the results obtained

with Group 7. In this group of rats 0.15 γ of estradiol per day administered for 3.5 days during the posttrauma period caused the same marked vaginal cornification that it did when given during the pretrauma period. This quantity of estradiol, however, not only failed to prevent the formation of placentomata but caused the formation of slightly larger ones than would have been formed with progesterone alone.

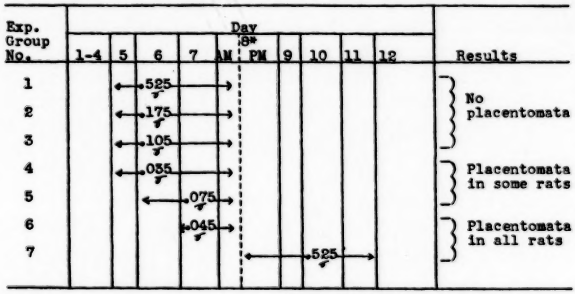


FIG. 1.—Effect of variation in amount of estrogen given during the pretrauma period on placentomata formation. The total length of the experiment was 12 days, with trauma of the uterus on the eighth day and autopsy on the thirteenth. Six-tenths RbU of progesterone was injected daily into each rat, and the estradiol (total dose shown) given in the amounts and on the days shown in the figure.

## DISCUSSION

In contrast to the augmenting action of estradiol on progesterone when the two hormones are given simultaneously (Allen, 1937; Korenchevsky and Hall, 1937; Pincus, 1937; Rothchild, Meyer, and Spielman, 1940), there is no evidence in the data presented here that estradiol augments progesterone when the latter follows the former, in so far as the size of the placentomata formed is concerned. Rather, the estradiol sensitization of the uterus to the action of progesterone would seem to be an "all-or-none" effect, since the size of the placentomata formed was the same in all groups of rats irrespective of the amount of estradiol used, while doses below 0.05 γ of estradiol gave completely negative results. These data are in accord with those obtained by McPhail (1934), which showed that variation in the amount of estrone given to immature rabbits before progesterone was injected had little or no effect upon the degree of uterine-gland proliferation obtained with a constant dose of progesterone.

In one sense, however, estradiol does augment the action of progesterone when given in this way. It has been shown here (Expt. II) and by Hisaw, Greep, and Fevold (1937) that it is not necessary, under certain conditions, to pretreat the uterus with estrogen for progesterone to be effective. That is, if a sufficiently large amount of progesterone (in these experiments, 2 RbU per day) is given during the pretrauma period, placentomata form, although the posttrauma dose is decreased to 0.6 RbU. However, when the animal is pretreated with estrogen, the minimum effective amount of progesterone can be decreased to 0.6 RbU for the pretrauma as well as for the posttrauma daily dose. The "augmentation" of progesterone by estradiol when the two are given in consecutive order would therefore appear to be such that, without affecting the size of the placentomata formed, estradiol decreases the effective amount of progesterone needed.

There exist in the rat three physiological states, differing from one another in certain respects, during which placentomata may be formed. These are pseudopregnancy, pregnancy, and lactation. Pseudopregnancy differs from pregnancy in its length, namely, 13 days as compared to 21–22 days, but resembles it in that the uterus in both conditions will form placentomata only when it is traumatized between the third and eighth day after estrus. Pregnancy resembles lactation in its length but differs from the latter in that placentomata may be formed at almost any time during lactation (Selye and McKeown, 1935; Lyon and Allen, 1938). A search of the literature has shown that little attention has been given to the question of why these differences in the length of the sensitive period of the rat's uterus exist. Selye and McKeown (1935) have described several experiments which ascribe the limiting factor in pregnancy to the influence of the developing placenta, while a suggestion has been made, without experimental evidence, by Lyon and Allen (1938) that the loss of sensitivity in pregnancy may be due to an increase in estrogen on the eighth to ninth day.

It is perhaps possible, by correlating the data presented in this paper with the known facts concerning ovarian and uterine physiology during pseudopregnancy, pregnancy, and lactation, to lay the basis for an explanation of these differences in the length of the sensitive period of the rat's uterus to trauma. It has been shown here that, under the influence of progesterone alone, the castrated rat's uterus remains sensitive to trauma for an indefinite period of time. On the other hand, amounts of estradiol which are lower than those required to inhibit the action of progesterone and which, moreover, are within the range which actually augment the action of the latter (Rothchild, Meyer, and Spielman, 1940) destroy this sensitivity effectively.

Might not the loss of sensitivity in the pseudopregnant rat therefore be due to an increase in the level of estrogen in the blood on or about the eighth day after estrus? This increase would probably come about as a result of the growth and beginning secretory activity of the follicles, which are destined to rupture on the thirteenth day and which therefore must begin their growth on the eighth or ninth day. In pregnancy the source of the estrogen may be ascribed to either the placenta or the ovary. As already mentioned, Selye and McKeown (1935) have described experiments which tend to prove that the placenta in the rat inhibits the growth of placentomata in the sterile horn of a unilateral pregnancy. However, the estrogen, to be effective in desensitizing the uterus, must be present by the eighth to ninth day after estrus. On the basis of certain unpublished data from this laboratory, a question can be raised as to whether the source of the estrogen in the pregnant rat is the placenta. We have found that the presence of placentomata in one horn of a castrate rat's uterus does not inhibit the formation of placentomata in the other. Since, on the ninth day of pregnancy in the rat, an implantation site differs from a placentomata only in the fact that the former contains a blastocyst while the latter does not, it is difficult to conceive of a wide difference in physiological activity existing between these two types of the same tissue. While it is not possible to discount the role of the placenta on the basis of this circumstantial evidence, we wish to point out another possible source of the estrogen, namely, the ovary. The secretion of estrogen from the ovary could occur during the period between the relaxation of the maintenance of luteal function by the pituitary and the assumption of this function by the placenta, which normally takes place between the ninth and twelfth day of pregnancy (Pencharz and Long, 1931 and 1933; Selye, Collip, and Thompson, 1933; Bergmann, 1934).

Lastly, in the lactating rat follicular growth is evidently held in abeyance for almost

the whole duration of lactation (Long and Evans, 1923), while luteal function continues. Thus practically no estrogen is secreted, and conditions are ideal for the indefinite maintenance of sensitivity of the uterus to trauma.

SUMMARY

When a constant dose of progesterone was administered to castrated rats, the size of placentomata formed was not affected by the amount of estrogen used to pretreat the animal.

Estrogen pretreatment was omitted entirely, and placentomata were obtained when a sufficient quantity of progesterone (2 RbU per day) was given to castrated rats. It was also shown that a large daily dose of progesterone is effective only in sensitizing the uterus to trauma. It will not cause the formation of placentomata in a uterus which has been improperly sensitized.

When daily progesterone injections were made, the sensitivity of the uterus to trauma was maintained for as long as 20 days after estrus, whereas, when small amounts of estradiol were given together with effective amounts of progesterone during the pre-trauma period, the sensitivity of the uterus was destroyed.

From these data a theory is formulated, which attempts to account for the duration of sensitivity of the uterus to trauma.

We wish to express our thanks to Dr. M. A. Spielman of the Department of Chemistry for the liberal supplies of noncrystalline progesterone, which made these experiments possible.

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# THE EFFECT OF HYPOPHYSECTOMY UPON THE RESPIRATORY QUOTIENT OF SNAKES[1]

(One figure)

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THE influence of the pituitary on many physiological processes has been the object of ever increasing experimentation. Because of the diverse functions of the pituitary gland, research may follow any of several paths. In the study of the role of the hypophysis in carbohydrate metabolism, much has been done on the usual experimental animals—rabbit, rat, mouse, guinea pig, dog, and pigeon.

A résumé of the results of recent work leads to the conclusion that, in mammals, carbohydrate metabolism is probably altered by two pituitary factors. The first—a diabetogenic hormone—exerts a hyperglycemic action, tending to antagonize the effects of insulin on blood sugar. The second—a glycotropic factor—apparently exerts a restraining influence upon the oxidation of glucose and may also be concerned with gluconeogenesis. Young (1938) tested the action of anterior-lobe extracts and found that the diabetogenic action is obtained only after extended injections, whereas the glycotropic action is, by comparison, quite rapid. The former requires several days, while the latter is effective within a few hours.

Very little has been done on the hypophysis of the snake in any way. Schaeffer (1933) observed the effect of hypophysectomy and thyroidectomy in these animals, reporting that the former operation caused a decrease in size of the gonads, adrenals, and thyroid. Shedding of the skin after hypophysectomy was often more frequent and irregular, and thyroidectomy indicated by its similar results that the effect on shedding was due to decreased thyroid activity subsequent to hypophysectomy.

Extensive research on the normal respiratory physiology of large reptiles has been pursued by Benedict (1932), who observed oxygen consumption, respiratory quotient, relation between environmental and body temperatures, and other factors involved in normal respiration and metabolism.

## SCOPE OF PRESENT WORK

The present work includes a series of R.Q. determinations on snakes before and after hypophysectomy. The former served to establish the normal R.Q. for the group of snakes and as self-controls, since all animals were subsequently hypophysectomized. After operation the second series was made, and the results of both series were compared statistically to determine if there was any significant difference between normal and

[1] Contribution from the Department of Zoölogy, University of Michigan.

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operated R.Q. values. The effect of hypophysectomy on absolute oxygen consumption was determined simultaneously. Replacement therapy with mammalian pituitary extracts was attempted. High carbohydrate feeding to the operated group was tried, in an effort to study changes in ability to metabolize carbohydrate.

Snakes were chosen as experimental animals for several reasons.

1. With a normal feeding interval of 3–4 weeks, they are in a "basal" state for 2–3 weeks, so that a greater proportion of the time is available for testing.

2. Because of its extreme flexibility, the snake occupies a small space for a given body weight, thus reducing dead air space in the apparatus. A 900-gm. snake will readily occupy a space in which a 350-gm. rat is accommodated with difficulty.

3. Owing to the low activity level of snakes, it is much easier to obtain a record of oxygen consumption uncomplicated by excess activity.

4. Concomitant with low activity, as well as the expected lower metabolic rate in poikilothermic animals, the oxygen consumption is low, so the snake is preferable to smaller reptiles, such as lizards.

5. Hypophysectomy is a relatively simple operation because of the mandibular articulation, and the slight tissue trauma minimizes postoperative effects. There is also a high resistance to infection in the mouth.

6. The pituitary in reptiles lends itself beautifully to operation. The infundibular stalk is long and slender and directed backward from the forebrain. The *pars anterior* is only very loosely connected with it, if at all, and is actually posterior and ventral to the remainder of the gland. In the snakes there is no *pars tuberalis* (De Beer, 1926). With such loose attachment of the *pars anterior*, its separation can be accomplished with relative ease.

## TECHNIQUES EMPLOYED

A. *General principles.*—The food of the snake is primarily protein, and an R.Q. somewhat lower than that of man might reasonably be expected after feeding. This quotient will soon decrease to fat level and remain there throughout the rather long fasting period. The maintenance of a normal blood sugar level soon depends upon conversion from non-carbohydrate sources, since the carbohydrate stores are depleted in a few days (Benedict, 1932). The glycerol of fat probably is one of the main sources of glucose, and the fat thus metabolized must exert a predominant influence upon the R.Q. level.

With the relatively small quantities involved in the respiratory activities of the snake, a closed-circuit method is more desirable for measuring them. The apparatus absorbs carbon dioxide in weighed soda lime tubes and graphically records the volume changes due to oxygen consumption. With these values the R.Q. can be calculated.

B. *Animal chamber.*—The snake, confined in a cylindrical wire-mesh basket having a hinged top and small side entrance covered by a sliding gate, is placed in the chamber proper—a copper container $4\frac{1}{2}$ inches high and 6 inches in diameter. The hinged cover of the latter closes into an oil-filled moat surrounding the rim of the chamber. Air enters through a tube in the bottom and passes out through a similar tube in the cover. A baffle under the cover diverts air to the periphery, assuring more complete circulation throughout the chamber. Air temperature of the chamber is determined by a thermometer (0.1° C. graduations) sealed in so that the outgoing air surrounds the bulb. The entire chamber is immersed in a water bath to within 1 inch of the top, with 6 liters of water providing close stabilization of internal temperature.

C. *Air impeller.*—Two cylindrical bells, 1½ inches in diameter and 6 inches in length, operate in their respective oil-filled shells, which have central tubes projecting above the surface of the oil. The bells are suspended from a grooved wheel by a cord which raises and lowers them reciprocally. The wheel is given a reciprocating motion through an arm attached eccentrically to a larger rotating wheel, which is belt driven by a variable-speed motor. Hydrostatic displacement thus alternately draws in and expels air as the bells move up and down.

D. *Valves.*—The conventional rubber "flutter" valves allow the air to pass in only one direction. They are inclosed in housings having outlets and inlets so arranged that, in conjunction with the air-impeller unit, they maintain a unidirectional flow of air.

E. *Absorption tubes.*—The apparatus employs two sets, each consisting of two large glass tubes, the first filled with calcium chloride and the second filled half with soda lime and half with calcium chloride. One set is used to establish equilibrium in the system before the actual test, then the other is connected in place of the first, to determine gravimetrically the carbon dioxide absorbed during the test run.

F. *Spirometer.*—A light bell suspended in an oil-filled shell with a central tube comprises the spirometer. It is connected to the system so that uniform internal pressure is maintained, thus actually responding to volume changes. The bell size is such that 1 mm. of vertical movement is equivalent to 0.5 cc. The bell is delicately counterbalanced over a precision pulley by a silk thread attached to a counterweight. The latter bears an arm with an ink-writing pen, so that movements of the bell yield a continuous kymograph record of oxygen consumption. Contact of the pen with the paper is insured by twisting the thread to produce a moderate amount of torsion.

G. *Apparatus.*—The apparatus as a whole is shown in Figure 1. The air impeller, with the valves, is interposed between the absorption tubes and chamber and can be controlled to provide unidirectional air flow through the system at a rate of from 500 to 2,000 cc., or more, per minute. All joints are carefully sealed against leakage, either by winding with wire or rubber bands or by lanolin coatings. An inherent characteristic of the apparatus, tending to minimize leaks, is that with slow-moving, large-volume displacements actual pressure variations, equally positive and negative, are less than 0.5 cm. of water pressure. This places no strain on joints such as would be encountered with a conventional motor blower or pump.

## ANIMALS USED

A. *General.*—The snakes used were of two species—*Pituophis sayi sayi* (*Schlegel*), the yellow bull snake, from Kansas; and *Elaphe guttata*, the corn snake, from Florida. Both species were generally tractable and amenable to handling. Frequent handling at times other than for actual testing conditioned them and minimized excitement due to similar manipulation previous to actual experimentation.

B. *Care.*—All snakes were kept in the same room, two to a cage, with winter-day temperature variations between 21° and 24° C. and never lower than 18° at night. In summer the variation was higher than this, but in all probability less than that encountered in the natural habitats. Apparatus temperature was approximated to that of the room, eliminating possible effects of altering the body temperature during testing.

Water was freely accessible at all times, and each animal was fed one or two mice every 3–4 weeks. They were disturbed as little as possible for 2 days after feeding, as excitement caused regurgitation of partly digested food by the more nervous snakes. They were all kept in good nutritional condition without the necessity of forced feeding.

Judging by the time of defecation of undigested detritus, as well as by the corroborative evidence of a return of the R.Q. toward fat level, the food was digested in 3–5 days. Benedict (1932), working with larger snakes, found that on a normal protein diet the caloric output returned to a basal level in a similar period. On the basis of the above-mentioned criteria, an interval of 6 or 7 days after feeding was considered ample to assure basal conditions for testing.

C. *Nutritional considerations.*—For a normal R.Q. of the fasting state the animal is assumed to be drawing its energy from fat stores. In mammals this condition is generally

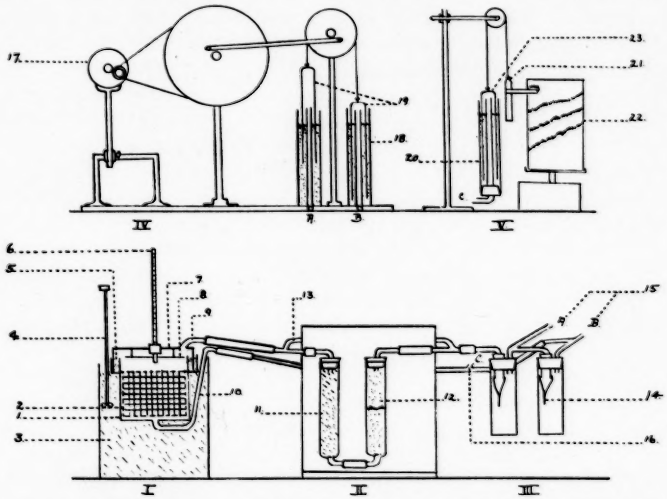


FIG. 1.—*I*, Animal chamber unit: *1*, chamber proper; *2*, wire basket for animal; *3*, water bath; *4*, water stirrer; *5*, oil moat for lid; *6*, thermometer; *7*, lid; *8*, baffle; *9*, outlet tube; *10*, inlet tube. *II*, Absorption tubes: *11*, calcium chloride tube; *12*, weighed tube, soda lime below, calcium chloride above; *13*, lead to duplicate set of tubes (not shown). *III*, Valves: *14*, rubber flutter valve; *15*, leads to air impeller (connect to *A* and *B* on impeller); *16*, lead to spirometer. *IV*, Air-impeller unit: *17*, stirring motor, variable-speed cone drive; *18*, shells filled with mineral oil; *19*, reciprocating bells; *A* and *B*, leads connecting with valves. *V*, Spirometer unit: *20*, shell filled with mineral oil; *21*, counterbalance with ink-writing pen; *22*, kymograph; *23*, spirometer bell.

regarded as obtaining after food has been withheld for about 18–24 hours. After this time, the stores of glycogen are virtually depleted, and thereafter the animal subsists on its fat stores, as indicated by a lower R.Q.

Benedict (1932) observed that tortoises, on a diet high in carbohydrate, maintained an R.Q. level well above 0.7 for as much as 8 days after feeding. However, the principal food of snakes is protein; and for this reason it is quite unlikely that they have large glycogen storage, though there is no direct experimental evidence to refute or corroborate this conclusion. Its plausibility may be inferred from R.Q. results, which indicate a return to a prefeeding basal level in considerably less time than that required by the tortoise. In addition, fecal expulsion should preclude the possibility of further absorption of protein or its conversion into fat or carbohydrate from food sources. Therefore, any changes taking place are much more likely to be of a catabolic nature, with fat break-

down predominating. This will continue to predominate until all fat stores have been depleted—a condition not occurring in the present series, as shown by the presence of fat deposits on post mortem examination. The practice of feeding at 3–4-week intervals was deemed adequate, inasmuch as all animals showed a progressive weight and size increase.

In large snakes the effect of ingestion of protein has been studied by Benedict (1932). The so-called specific dynamic action of protein in mammals is well established and increases the metabolic rate from 30 to 40 per cent. A radically different situation exists in the snake. Shortly after food has been ingested, the heat output increases as much as 300 per cent. However, this is only temporary; and, in terms of total increment, Benedict found the cost of digestion of protein to be approximately 20 per cent, appreciably less than the cost for warm-blooded animals.

It can thus be seen that there is a nutritional economy in the digestion of a normal reptilian diet. Added to this is the general low level of metabolism common to poikilothermic animals. A fasted state of 3 or 4 weeks is a normal occurrence in the snake's existence and does not violate its normal physiological state in any manner which could be construed to introduce factors unnatural to the living habits of the animals used.

D. *Testing techniques.*—The snake to be used must have been fed no less than 5 days previously. The tube with the soda lime and calcium chloride is weighed to an accuracy of 1 mg. and connected into the circuit, but blocked so that air will pass through the duplicate set. The basket with the animal is placed in the chamber, the cover closed, and the air impeller started. Several minutes are allowed for attaining equilibrium in the system, while sufficient oxygen is added to fill the spirometer. At the start of the actual test the air circuit is shunted to the weighed tube, the start noted on the kymograph record, and the internal air temperature recorded.

The experiment may be continued as long as the spirometer capacity allows. Though this is only 60 cc., the oxygen consumption of the snake is so low that as much as 4 hours may elapse in using this amount. At the termination of the test the final chamber temperature is noted and the end indicated on the kymograph record. After the animal is removed from the chamber, the absorption tube is weighed and the barometric pressure recorded. If more than one test is desired, the air is shunted to the duplicate tubes, with the free leads blocked to keep the system closed. Without stopping the air impeller, oxygen is added and the system allowed to reach equilibrium. Meanwhile, the experimental tube can be weighed, reconnected to the system, and another test started within 5 minutes. By this method it is possible to obtain from three to six tests per day, since the snake is entirely passive and not affected by the confinement. Such a procedure also allows a check on possible diurnal variations, although these were found to be practically negligible.

In calculation, the volume of oxygen consumed is determined by measurement of the kymograph record and weight of carbon dioxide converted to volume at standard temperature and pressure. Oxygen volume is corrected to standard conditions, and a further correction is made if the temperature changed during the test. A change in temperature of 0.1° C. was found to be equivalent to 0.9 cc. of volume change, to be added or subtracted in accord with the direction of change in temperature.

## NORMAL SERIES

*Respiratory quotients.*—The snakes were first used to establish a normal fasted R.Q. level and, with two exceptions, were tested several times to determine individual mean

respiratory quotients. The appearance of idiosyncrasies in the apparatus under operating conditions eliminated earlier results until all sources of error had been removed.

The testing extended from August, 1938, to May, 1940. Periodic advancement of individual animals from control group to operated group was made, as averages for each warranted the assumption that a satisfactory normal series had been obtained. In all, over one hundred individual determinations were made—a number deemed sufficiently large to average as a basis for the mean R.Q. Individual means ranged from 0.678 to 0.713, the mean of these being 0.699 (Table 1).

At first sight it might appear that some tests should be discarded because of their abnormally low values, but this apparent discrepancy was also encountered by Benedict (1932), who found some values at first attributed to technical error but later accepted, because, as experimentation progressed, it became evident that they occurred too frequently to be due to error. Such low values usually occurred when the environmental temperature was low, and at 18° C. he was able to maintain an R.Q. of 0.60 for several days in succession, with minimal values of 0.52. The present series of experiments did not embrace temperatures quite so low as this, and accordingly the minimal value, 0.56, also was above his lowest R.Q.

TABLE 1

NORMAL RESPIRATORY QUOTIENTS

| Snake | Weight (Gm.) | No. of Tests | Maximum R.Q. | Minimum R.Q. | Mean R.Q. | Sigma of Mean | Snake | Weight (Gm.) | No. of Tests | Maximum R.Q. | Minimum R.Q. | Mean R.Q. | Sigma of Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P*1... | 415 | 13 | 0.760 | 0.630 | 0.709 | 0.011 | *P*9... | 310 | 10 | 0.736 | 0.632 | 0.700 | 0.011 |
| *P*3... | 390 | 7 | 0.820 | 0.560 | 0.698 | 0.065 | *E*1... | 535 | 7 | 0.797 | 0.641 | 0.702 | 0.019 |
| *P*4... | 470 | 5 | 0.727 | 0.654 | 0.704 | 0.010 | *E*2... | 310 | 19 | 0.810 | 0.600 | 0.703 | 0.014 |
| *P*5... | 135 | 6 | 0.750 | 0.564 | 0.687 | 0.028 | *E*3... | 210 | 8 | 0.715 | 0.691 | 0.701 | 0.003 |
| *P*6... | 450 | 5 | 0.760 | 0.600 | 0.700 | 0.025 | *E*4... | 175 | 6 | 0.729 | 0.667 | 0.693 | 0.008 |
| *P*8... | 350 | 7 | 0.715 | 0.664 | 0.698 | 0.007 | | | | | | | |

At present there is no adequate explanation for this phenomenon. The possibility of nitrogen's being given off was ruled out by Krogh (1906) and Oppenheimer (1906, 1907). Carbon dioxide retention was suspected, but Benedict calculated that retention sufficient to cause such R.Q. decreases would cause a weight change, and such was not detectable in his animals. Furthermore, the stored gas would eventually be released when the temperature rose, increasing the R.Q. above normal. This, too, did not occur.

The problem remains unanswered, and much remains to be learned about actual intermediate metabolic processes in reptiles; but the occurrence of low R.Q. values can be considered an actuality and not an artefact or technical error.

## HYPOPHYSECTOMIZED SERIES

As mentioned earlier, the snake is admirably suitable for hypophysectomy. The *sella turcica* is a well-defined depression in the sphenoid bone and is readily located in the roof of the mouth. The jaw is opened at right angles to the body, and, after a midline incision through the oral mucosa, the bone is drilled with dental burrs. The anterior lobe is removed through the hole by suction with a fine glass pipette. Healing is rapid, with no incidence of infection. Intraperitoneal nembutal eliminates the difficulties of inhalation anesthesias incident to any oral surgery.

*Postoperative conditions.*—The operated animal is isolated for several days in a sterilized cage to avoid infection—which was never encountered—and then returned to its own cage. At least a week intervened between operation and testing.

Techniques on the hypophysectomized animals were the same as for normals. Oxygen consumption decreased, so that individual tests usually lasted longer; the fall in metabolic rate was approximately 25–40 per cent. As reported by Schaeffer (1933), the subsequent shedding shortly after operation served to check on the success of pituitary removal. Feeding was the same, and all animals accepted food readily. Since snakes are normally rather inactive, it would be difficult to determine if they were less active after operation. There was no apparent loss in speed or proficiency in catching mice.

One hundred and twenty-two tests on twelve animals showed mean R.Q. values ranging from 0.775 to 0.793 with a group mean of 0.778. The thirteenth snake showed no significant variation from the normal R.Q. and was found upon examination of histological sections of the pituitary region to have been incompletely hypophysectomized.

TABLE 2

HYPOPHYSECTOMIZED SERIES

| Snake | No. of Tests | Maximum R.Q. | Minimum R.Q. | Mean R.Q. | Sigma of Mean | Snake | No. of Tests | Maximum R.Q. | Minimum R.Q. | Mean R.Q. | Sigma of Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *P*1..... | 17 | 0.853 | 0.707 | 0.785 | 0.011 | *P*10.... | 10 | 0.797 | 0.770 | 0.780 | 0.003 |
| *P*4..... | 6 | 0.643 | 0.747 | 0.695 | 0.015 | *P*11.... | 10 | 0.788 | 0.770 | 0.778 | 0.003 |
| *P*5..... | 6 | 0.816 | 0.762 | 0.789 | 0.006 | *E*1..... | 13 | 0.849 | 0.748 | 0.793 | 0.008 |
| *P*6..... | 10 | 0.807 | 0.762 | 0.781 | 0.004 | *E*2..... | 6 | 0.797 | 0.745 | 0.775 | 0.007 |
| *P*7..... | 11 | 0.835 | 0.752 | 0.786 | 0.007 | *E*3..... | 7 | 0.806 | 0.767 | 0.779 | 0.006 |
| *P*8..... | 14 | 0.814 | 0.764 | 0.779 | 0.003 | *E*4..... | 10 | 0.826 | 0.757 | 0.782 | 0.007 |
| *P*9..... | 9 | 0.795 | 0.767 | 0.780 | 0.008 | | | | | | |

When an experimental series on an animal was completed, it was killed, and the brain was removed, fixed, and sectioned, with particular care in the removal of all tissue from the *sella turcica*. Serial sections were examined for remnants of anterior-lobe tissue. With two exceptions, no significant amount was present. In *Elaphe* 2, there was a small proportion of scattered rests, and *Pituophis* 4, as previously cited, showed a considerable portion of active tissue. The former had the lowest mean R.Q. of the successfully operated series, and the latter had a normal R.Q. The correlation between R.Q. results and histological findings is evident (Table 2).

*Replacement therapy.*—Attempts were made, using mammalian pituitary extracts, to restore normal R.Q. levels. Although repeated massive doses were injected, no significant reduction in the R.Q. was obtained. This possibly is due to some interclass difference in pituitary hormones, so that mammalian hormones do not function properly to restore normal pituitary physiology in reptiles. That the operated results were due to trauma elsewhere in the brain is unlikely, because the peculiar anatomy of the snake pituitary minimizes such a possibility.

*Carbohydrate feeding.*—Feeding of killed mice, into which large amounts of concentrated carbohydrate solutions had been injected, was carried out in an attempt to study carbohydrate utilization by the snakes. Results were so variable that no conclusive evidence could be obtained.

## DISCUSSION

Examination of the statistical data from the normal and operated series (Table 3) shows that the results are significant, with critical ratios more than three times the standard error of the difference.

As demonstrated by Houssay (1936), Schaeffer (1933), Fisher, Russell, and Cori (1936), and many others, hypophysectomy inevitably influences other endocrine glands. The thyroid is principally affected in causing a lower metabolic rate. Schaeffer reported a decrease in size of the thyroid by as much as one-third following hypophysectomy in snakes. However, this does not explain the altered R.Q., despite the decreased metabolic rate. The antagonism between pituitary hormones and insulin—a factor affecting carbohydrate metabolism—was shown by Houssay and Magenta (1927) to be independent of the thyroid.

TABLE 3

STATISTICAL SUMMARY OF RESPIRATORY QUOTIENTS

| Snake | Mean Normal | Mean Operated | Difference | $\sigma_m$ (Normal) | $\sigma_m$ Operated | Sigma Difference | Critical Ratio |
|---|---|---|---|---|---|---|---|
| *P*1 | 0.709 | 0.785 | +0.076 | 0.0109 | 0.0109 | 0.0154 | 4.9 |
| *P*4 | 0.704 | 0.695 | −0.009 | 0.0099 | 0.0153 | 0.0182 | 0.5 |
| *P*5 | 0.687 | 0.789 | +0.102 | 0.0266 | 0.0066 | 0.0274 | 3.7 |
| *P*6 | 0.700 | 0.781 | +0.081 | 0.0255 | 0.0045 | 0.0259 | 3.1 |
| *P*7 | 0.700 | 0.786 | +0.086 | 0.0196 | 0.0069 | 0.0209 | 4.1 |
| *P*8 | 0.698 | 0.779 | +0.081 | 0.0070 | 0.0034 | 0.0076 | 10.7 |
| *P*9 | 0.700 | 0.780 | +0.080 | 0.0106 | 0.0030 | 0.0114 | 7.0 |
| *P*10 | 0.700* | 0.780 | +0.080 | 0.0196* | 0.0027 | 0.0198 | 4.0 |
| *P*11 | 0.700* | 0.778 | +0.078 | 0.0196* | 0.0017 | 0.0194 | 4.0 |
| *E*1 | 0.702 | 0.793 | +0.091 | 0.0196 | 0.0081 | 0.0212 | 4.3 |
| *E*2 | 0.703 | 0.775 | +0.072 | 0.0140 | 0.0076 | 0.0159 | 4.5 |
| *E*3 | 0.701 | 0.779 | +0.078 | 0.0031 | 0.0060 | 0.0067 | 11.6 |
| *E*4 | 0.693 | 0.782 | +0.089 | 0.0083 | 0.0074 | 0.0106 | 8.4 |

* Average group mean, used in absence of individual normal series.

Houssay and associates found a factor in the pituitary complex having an action on blood sugar antagonistic to insulin and exerting a diabetogenic action in the depancreatized animal. Geiling *et al.* (1927), Young (1936), Corey and Britton (1937), Long and White (1938), and many others have definitely established the existence of this diabetogenic factor.

Fisher, Russell, and Cori (1936) showed that hypophysectomized rats oxidize glucose more rapidly than do normal rats, and this in turn accounts for the rapid decrease in blood sugar as well as in liver and muscle glycogen in the fasted hypophysectomized animals. This suggests that the pituitary tends to restrain carbohydrate oxidation and enables the maintenance of glycogen storage during the fast. Substitution therapy with anterior-lobe extract depresses the R.Q. to the normal fasting level and retards the disappearance of liver glycogen.

Russell and Bennett (1936) extended and confirmed these results; and Soskin *et al.* (1935), Weinstein (1939), Corey and Britton (1937), and Young (1936, 1938) used dogs similarly. Soskin approached the problem from the angle of glyconeogenesis. It was felt that the pituitary affected the formation of blood sugar rather than its usage. In the fasted animals there is no exogenous source of carbohydrate for glycogenesis, and so

glucose must be derived from protein and fat by glyconeogenesis. Hypophysectomized dogs showed failure of glyconeogenesis when, together with control animals, they were fed fat. The controls had a long survival rate, while the operated series survived no longer than if they had not been fed, indicating that they were unable to spare body protein by the utilization of ingested fat for glyconeogenesis.

Soskin, Levine, and Heller (1939) postulate the role of the pituitary by three possible routes—(*a*) directly, through the diabetogenic factor, (*b*) through a possible effect on the thyroid, which may alter protein-amino acid catabolism, thus secondarily influencing protein glyconeogenesis, and (*c*) a possible influence, still unexplained, on glyconeogenesis from fat.

Whether it is fully justifiable to apply these same theories to the snake is uncertain. This animal subsists almost entirely on fat and protein, except for the relatively small amounts of carbohydrate in the tissues of the animals eaten. Benedict (1932) states that snakes apparently have large fat storage, as indicated by a continued maintenance of a fat R.Q. for many weeks of fast.

In the present series, examination of internal organs revealed fat deposits in all cases, precluding the possibility of fat depletion causing protein breakdown after hypophysectomy. There was no apparent difference in the nature of these deposits in normal and operated animals, since they were mainly in the form of plaques in and around the mesenteries in both groups.

The experimental findings with the snakes would easily fit the theories advanced by Soskin, since the fat of the operated animals was apparently not adequately utilized and the higher R.Q. results could be attributed to the increased catabolism of protein for glyconeogenesis. If this were true, then the snake pituitary would seem to have functions similar to the mammalian pituitary, or at least possess a remarkable parallelism.

On the other hand, there might be some process of intermediate metabolism of fat or protein of a type as yet unknown in higher forms. Benedict stressed the lack of knowledge about the intermediate processes of metabolism in poikilothermic animals; and, until this has been studied in detail, the exact mechanism of the influence of hypophysectomy upon metabolism in snakes and its consequent reflection through the R.Q. will remain a theoretical assumption of similarity in the pituitary functions of snakes and mammals.

## SUMMARY

1. One hundred and thirty R.Q. determinations on thirteen hypophysectomized snakes indicate that removal of the anterior lobe causes an increase in the R.Q. of fasted animals above that of fat metabolism. Such an increase is probably brought about by a decrease or loss of metabolic control by factors normally present in pituitary secretions.

2. Replacement therapy, attempted with mammalian pituitary extracts, gave no indication of return to a normal R.Q. This possibly was due to interclass variations in pituitary hormones.

3. Feeding experiments involving administration of carbohydrate were attempted, with results which could not be considered significant.

4. In order to carry out experimentation, apparatus was designed for studies on respiratory metabolism of small animals, in combination with an original type of air impeller, whose characteristics minimize pressure gradients and their attendant possibilities of leakage.

5. A simple approach to hypophysectomy was devised, eliminating many complicating factors normally present in the mammalian operation.

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# THE MORPHOGENESIS OF DOWN FEATHERS WITH SPECIAL REFERENCE TO THE DEVELOPMENTAL HISTORY OF MELANOPHORES[1]

(Six text figures; four plates)

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## I. INTRODUCTION

PIGMENT cells from various breeds or species of birds can be introduced into feather germs of host embryos of different breeds by transplanting potential melanophores into the base of the right wing bud of host embryos of 70 hours' incubation (Willier and Rawles, 1940). These implanted melanoblasts migrate into host epidermis and bring about a reproduction of the color or color pattern characteristic of the donor breed or species. Thus, if Barred Plymouth Rock melanoblasts are transplanted to White Leghorn hosts, the down feathers surrounding the transplantation site develop the typical black down coloration of the donor, and the juvenile feathers produced in these follicles exhibit a typical barred pattern except for certain modifications resulting from influences present in host feather germs (Willier, 1941).

Several lines of evidence suggest that "epidermal" melanophores also have an extrinsic origin in normal development. (1) Skin ectoderm from wing buds of Barred Rock embryos incubated less than 79 hours contributes no pigment cells when implanted into host wing buds, whereas skin ectoderm from wing buds of embryos 80 hours and older yields abundant melanoblasts which invade host feather germs (Willier and Rawles, 1940). This indicates that melanoblasts do not enter donor wing-bud epidermis before approximately 79 hours. (2) That melanophores originate even outside of the wing bud is indicated by two experiments: (*a*) Wing-bud mesoderm from Barred Rock embryos incubated less than 72 hours yields no melanophores when transplanted, whereas mesoderm from embryos 94 hours and older contains many melanoblasts (Watterson, 1938). (*b*) If the limb bud of a 60- to 70-hour Barred Rock embryo is isolated at the level of the intermediate cell mass and implanted into the coelom of a host embryo, it will develop white down feathers, i.e., the graft contains no potential melanophores. However, if the limb bud is isolated adjacent to the neural tube and then implanted into the coelom, black feathers develop; i.e., the graft contains potential melanophores whose source must be adjacent to the neural tube (Eastlick, 1939). (3) That melanophores arise only from the neural crest has been demonstrated by Ris (1941). It is thus apparent that the seat of origin of melanophores is the neural crest, from which they migrate into the limb buds and finally into the epidermis and feather germs.

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The general purpose of this paper is to trace by means of histological methods the behavior of Barred Rock melanophores from the onset of their migration into the wing bud until the end of incubation. Special attention will be given to several specific problems. The first problem deals with the time when the precursors of melanophores can first be recognized, when they begin to migrate into the epidermis, and how long this migration continues. This includes the manner of their incorporation into those feather germs which produce black down feathers, as well as their failure to enter those which form the white feathers commonly found on the wing tips and ventral surface of Barred Rock chicks. The second problem is concerned with the differences which arise during the differentiation of male and female melanoblasts into definitive melanophores. Are there sex differences in the quantity of melanin produced, such as might be anticipated from the well-known sexual dimorphism of the juvenile and adult plumage of the Barred Rock? If such differences exist, can they be correlated with differences in the growth rate of feathers in males and females? Such a correlation might be expected, since it has been demonstrated that in juvenile and regenerating feathers the amount of pigment deposited depends upon the growth rate of the feather. Finally, a correlation is attempted between the distribution and behavior of melanophores and the morphogenetic changes taking place within the feather germ. Such a study involves observations on the mode of deposition of pigment into epidermal cells, the locus of melanophore proliferation, and the ultimate fate of the melanophores.

## II. HISTOLOGICAL EVIDENCE FOR MIGRATION OF MELANOBLASTS INTO WING-BUD EPIDERMIS

In order to study the migration of melanoblasts, transverse serial sections, cut at 7 $\mu$ and stained lightly with iron haematoxylin, were prepared through the wing-bud level of Barred Rock embryos at hourly intervals from 75 to 96 hours of incubation and at daily intervals through the wings of 4- to 9-day embryos. These preparations were supplemented with whole mounts of the skin from the outer margins of the ulnoradial region (site of future secondary remiges). The number of cells overlapping the dermoepidermal boundary was counted for each pair of wing buds in at least two embryos of each stage. After the appearance of melanin granules the number of dividing melanophores in the wings of each embryo was determined.

The epidermis of wing buds of 75- to 80-hour embryos consists of a single layer of cuboidal cells adjacent to the dermis, covered with a layer of flattened epitrichial cells. From the eightieth to the ninety-first hour cells are observed which, according to the position of their nuclei, appear to lie partly in the dermis and partly in the epidermis (Pl. I, Fig. 7). The number of such dermoepidermal cells is always small, ranging from two to five per pair of wing buds at 80 hours' incubation to a maximum of seven or eight at 82 hours, and then decreasing to one or none at 90 and 91 hours, respectively. Typically, they contain very little cytoplasm and are characterized by a more or less elongated nucleus, which usually contains a single enlarged nucleolus. There is a decided tendency for the nucleolus to occupy that part of the nucleus which lies in the dermis. Their long axis usually lies at approximately right angles to the epidermis, and they are found with varying proportions of their length protruding into the epidermis. The only histological criterion which could possibly distinguish them as potential melanophores is the enlarged nucleolus, which Rabl (1894) and Champy (1935) have observed to be characteristic of melanophores during active synthesis of pigment. The best evidence that these cells are

potential melanophores is the fact that their first appearance coincides with the earliest stage at which Willier and Rawles (1940) can obtain a donor-colored area by implanting wing-bud epidermis into the base of host wing buds.

The epidermis of wing buds of embryos 91 hours and older contains, in addition to the epitrichial and cuboidal cells present earlier, a third cell type, an elongated cell which tends to lie roughly parallel to the surface of the skin. Presumably, these cells are the melanoblasts which have just migrated into the epidermis. They are apparently identical with the *Mutterzellen der Melanophoren* described by Kawamura (1933 and 1934).

The invasion of wing-bud epidermis by melanoblasts appears to be restricted to the short period between 80 and 91 hours of incubation. In exceptional cases melanophores have been observed in older stages (9 days) with their cell bodies in the dermis and a cell process extending into the epidermis. Thus, processes of melanophores can still penetrate into the epidermis, although there now appears to be no tendency for the entire cell to do so. A much more common picture is to find a melanophore process which is directed toward the epidermis reflected at the dermoepidermal boundary in such a way that it lies along the underside of the epidermis for some distance. These observations are in agreement with the *in vitro* studies of Hamilton (1940) to the effect that "extensive migration of pigment cells is apparently confined principally to the melanoblast stage, for as soon as granules are differentiated, movements of the cells are confined to protrusion and retraction of processes with little shifting of position" (p. 529).

Melanoblasts in the wing-bud epidermis begin the formation of melanin between 7 and 7½ days' incubation and thus become definitive melanophores. By 8 days the epidermis of the outer margin of the ulnoradial level of the wing contains rather uniformly distributed melanophores (Pl. I, Fig. 8). The large number of melanophores now present must indicate, in view of the small number of dermoepidermal cells observed earlier, that a rapid postmigratory proliferation has occurred, either during the melanoblast stage or perhaps later, after melanin formation has begun. No data have been obtained on the frequency of melanoblast mitoses, owing to the obvious difficulty in identifying these cells during their division. Data have been obtained for wing-bud epidermis of embryos of such an age that melanophores are definitely identified in section by their melanin content. The number of melanophores in mitosis at this time is considerable at the ulnoradial level of the wing.

Kerbert (1876) and Rosenstadt (1897) described isolated accumulations of pigment granules in the chick epitrichium. According to their observations, processes of melanophores never penetrate into the epitrichial layer, and, accordingly, they concluded that this pigment arose *in situ*. However, in wing-bud epidermis elongated melanophores may remain entirely within the Malpighian layer and its derivatives, or they may be surrounded partly by these and partly by epitrichial cells. Moreover, cells in the epitrichium which contain pigment are not typical flattened epitrichial cells but are more elliptical, contain an enlarged nucleolus, and are in every respect identical with the young melanophores observed among Malpighian cells. Presumably, then, those cells of the epitrichial layer which do contain pigment are melanophores which have moved into this surface layer of the epidermis. There is thus no reason to believe that epitrichial cells are capable of independent synthesis of melanin.

## III. DEVELOPMENT AND PIGMENTATION OF SECONDARY FLIGHT FEATHERS DURING INCUBATION

### 1. MATERIALS AND METHODS

Transverse serial sections of the ulnoradial region of wings of 7- to 10½-day Barred Rock embryos were prepared at 3-hour intervals. These provide longitudinal sections through young feather germs. Transverse serial sections of secondary feather No. 2 (see Warren and Gordon, 1935) were obtained at 3-hour intervals from the ninth to the eleventh day of incubation, and both transverse and longitudinal sections were prepared at daily intervals from the eleventh day until hatching. All sections were cut at 7 $\mu$ and were stained lightly with iron haematoxylin. In addition, whole mounts of wing skin taken at close intervals from the sixth to the tenth day were studied. Pieces of skin removed from the outer margin of the ulnoradial region were transferred in a drop of Locke's solution to a cover slip. The dermis was placed against the glass, and the skin was flattened by carefully withdrawing the saline. Fixation was in Allen's B-15. After removing the skin from the cover slip, it was cleared and mounted unstained. Whole mounts were made at 3-hour intervals from unstained feathers of 11- to 13-day embryos, and at daily intervals from the thirteenth day until hatching. Split feather preparations (Lillie and Juhn, 1932) were obtained at 3-hour intervals from embryos of 9⅝–12 days' incubation and at daily intervals from then until hatching. The procedure involved in making split preparations of down feathers was rather delicate. A steel needle with a ground cutting edge was inserted into the pulp from the base of the feather germ, and the cutting edge was held firmly against the inside of the epidermal cylinder throughout the length of the feather germ. The ground edge of a second steel needle was held transverse to the long axis of the feather germ, was pressed down firmly on the epidermis overlying the needle in the pulp, and was moved back and forth along the length of the feather germ. In this way the epidermal wall and the sheath were severed cleanly with no tearing or distortion. The cut feather germs were transferred in a drop of Locke's solution to a cover slip, were placed with the pulp against the glass, were spread flat by carefully withdrawing the saline, and were fixed in Allen's B-15. The pulp adheres to the cover slip throughout further treatment; accordingly, the unstained preparations, after clearing and mounting, are viewed from the pulp surface. Such preparations proved extremely helpful in interpreting the tangle of melanophore processes seen in sections. Completely formed down feathers were flattened and mounted dry. The flattened strand of pulp and overlying epidermal cells which is attached only to the base of the down feather was removed after hatching and then fixed, cleared, and mounted unstained. A chronological study of the development and pigmentation of the down and juvenile feathers was then made from these preparations.

### 2. ONSET OF FEATHER-GERM FORMATION

The outer margin of the ulnoradial region of the wing is characterized by an early condensation of dermal cells, forming a broad continuous dermal ridge. As early as 7¼ days there are indications of slight localized changes in the epidermis overlying this ridge, viz., a lengthening of cuboidal cells at intervals along the wing edge. Each such localized thickening has a diameter of from 105 to 119 $\mu$. By 7½ days this dermal band is partly separated into two longitudinal bands, the outer corresponding to the locus of remige formation, the inner to that of the major coverts (cf. Holmes, 1935). The elon-

gated epidermal cells begin to divide horizontally, and layers of "intermediate cells" thus appear between cuboidal and epitrichial layers, resulting in the formation of localized epidermal caps, within which melanophores are dividing rapidly and beneath which dermal cells are undergoing extensive proliferation, causing the epidermis to protrude outward.

During the earliest morphogenetic changes melanophores begin to disappear from the epidermis on the proximal side of each feather germ (Pl. I, Fig. 9). No scattered clumps of pigment granules are visible in these melanophore-free areas or spots. By the ninth day individual feather germs become visible as distinct elevations along the margin of the the wing (Pl. I, Fig. 10). The melanophores are enlarged and are distributed throughout the epidermis, except in those areas from which they began to disappear earlier. The melanophore-free spots become most sharply defined in wing feather germs of embryos of $9\frac{1}{8}$ days' incubation. The spot lies on the exact medial side of the axial feather germ (i.e., the feather germ at the junction of hand and arm), but in the successive secondary feather germs it becomes progressively shifted toward the outer surface of the wing in distoproximal order. The brief history of these spots is most readily followed in the tail region of 9-day embryos, where feather germs are present in several stages of development (Pl. I, Fig. 11). The outlines of the spot become visible even before the feather germ forms a distinct elevation. They become progressively more sharply defined in larger and larger germs until suddenly they fade away and disappear completely in the largest feather germs. These spots are obviously transitory in nature. They are clearly related to the symmetry of the bird, since on the appendages they always occupy the proximal side of the germs, whereas on the body proper they lie on the anterior side.

### 3. SEXUAL DIFFERENCES IN MELANIN PRODUCTION

By the tenth day the feather germ is filled with a complex network of pigment cells which appear to be distributed entirely at random; and, although each melanophore is packed with pigment granules, no pigment has been deposited outside its cell body as yet (Pl. II, Fig. 12). Consequently, the degree of blackness of feather germs at this time is due entirely to pigment stored within melanophores. There is a sharp contrast in the amount of melanin present in male and female feather germs. By the tenth day the dorsal feather germs of females are so much darker than those of males that embryos can be sexed with 100 per cent accuracy on this basis (classification checked by examining gonads; see Pl. I, Fig. 13). Wing feathers also reveal this sexual difference at a slightly later stage. However, sexual dimorphism in the amount of melanin in wing feathers is never as striking as in back feathers and consequently is never as reliable an index of the sex of the embryo. Sexing chicks by the intensity of coloration of feathers becomes difficult during the remainder of incubation; but, in general, pigmented feather areas of females appear jet black, whereas those of males have a grayish cast.

### 4. REARRANGEMENT OF MELANOPHORES

In embryos incubated $10\frac{1}{8}$ days the random distribution of melanophores begins to disappear (Pl. II, Fig. 14). They become regularly arranged in a series of longitudinal rows. This begins near the apex of the feather germ and spreads basally. It does not occur simultaneously throughout the circumference of the feather germ but begins dorsally and spreads ventrally.[2] By $10\frac{2}{3}$ days all melanophores in the distal two-thirds of the

[2] The terms "dorsal" and "ventral" correspond respectively to the outer and inner surfaces of the future feather vanes. They are defined in terms of juvenile and adult feathers, but they are also applicable to the feather germs of down feathers.

feather germ are definitely distributed into ten or eleven longitudinal rows, whereas in the proximal third they still show a random distribution (Pl. II, Fig. 15).

### 5. THE COMPLETED DOWN FEATHER

The conditions which lead to this redistribution of melanophores become evident upon an examination of the structure of the completed down feather (Pl. III, Fig. 17) and its projection onto the feather germ (text Fig. 2). Each feather follicle produces first a down feather and then a juvenile feather (Pl. II, Fig. 16). As each juvenile feather increases in length, its down feather is carried distally; and, after it has emerged from the follicle, it is ultimately broken off and lost. As seen in Plate III, Figure 17, each down feather consists of from ten to fifteen barbs radiating outward in all directions and upward from a horny cylinder, the calamus, which attaches them to the tip of a juvenile feather. Thus the completed down feather is a radially symmetrical structure. The distance from the base of the calamus to the apex of any barb is approximately $\frac{1}{2}$ inch, as Pernitza (1871) has observed.

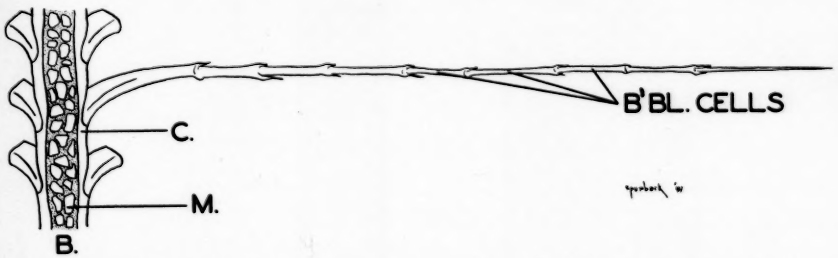


FIG. 1.—The histological structure of a down barb and one of its barbules (diagrammatic). *B.*= barb; *B'BL.*=barbule; *C.*=cortex; *M.*=medulla.

The barbs are roughly equal in length (Hosker, 1936), although there are frequent exceptions to this rule. Studer (1873) believed that one barb exceeds the others in size and importance, and Klee (1886) described two *Hauptstrahlen* for the down feather, but neither observation has been confirmed. Each barb is broad at its base and tapers gradually to a fine point at its apex. On the proximal two-thirds of its length each barb bears two rows of barbules placed diametrically opposite each other (Studer, 1873) and extended roughly at right angles to the barb. The barbules are very short near the base of the barbs, but just distal to the attachment of the barbs to the calamus they reach their maximum length. They then decrease in length in a proximodistal direction, gradually at first and then quite abruptly. Near the base of the down feather, barbule insertions are closely approximated along the length of the barbs and lie directly opposite one another. In more distal regions the interval between barbule attachments increases progressively, and their insertions on one side of the barb tend to alternate with those on the other. The number of barbules on each barb is quite variable and is never identical on the two sides of any one barb, as the representative figures (65-61, 85-74, 112-99) indicate.

Text Figure 1 represents diagrammatically the detailed structure of barbs and barbules. Each barb (*B.*) consists of large central medullary cells (*M.*) inclosed by an outer cortex (*C.*) of flattened cells, as Studer (1873) and Davies (1889) described. Each barbule consists of a single row of elongated cells (*B'BL. CELLS*) joined end to end (cf.

Wohlauer, 1902, and Strong, 1902). There are from eighteen to twenty such cells in the longer barbules (Pernitza, 1871). The basal cell of each barbule is broadly attached to the barb (Hosker, 1936), and the distal end of each is expanded to receive the narrow proximal end of the next distal cell (Pernitza, 1871; Studer, 1873; Hosker, 1936). Frequently these expansions are extended as one or two pointed processes (Jones, 1907). The barbules of one barb-vane[3] are not attached to those of adjacent barb-vanes, as in definitive feathers (Remak, 1855; Stieda, 1869; Jones, 1907). Accordingly, barbule cells of down feathers are little specialized and ordinarily taper gradually to a point (Pernitza, 1871). However, the distal cells of barbules located near the attached ends of barbs are sometimes considerably modified and exhibit numerous spines, similar to those described in ducklings by Lamont (1922).

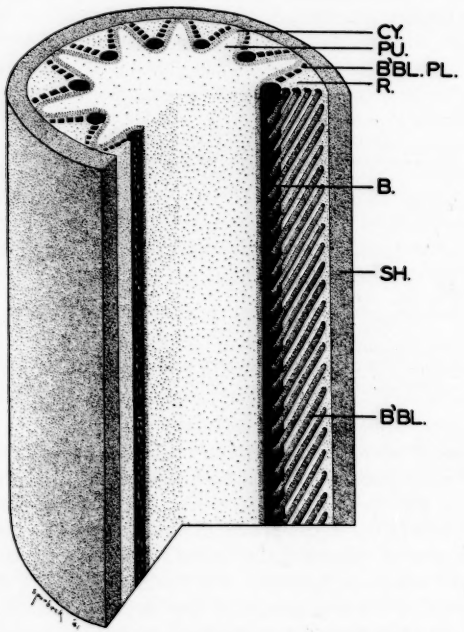


FIG. 2.—Schematic projection of a down feather onto the feather germ. *B'BL. PL.* = barbule plate; *CY.* = cylinder cell layer; *PU.* = pulp; *R.* = barb-vane ridge; *SH.* = feather sheath.

### 6. PROJECTION OF THE DOWN FEATHER UPON THE FEATHER GERM

If the component parts of each barb-vane are projected back upon the feather germ, they exhibit different spatial relationships from those observed in the definitive feather. Within the feather germ (text Fig. 2) each barb-vane is folded up in such a way that it forms a longitudinal barb-vane ridge (*R.*) projecting inward toward the pulp (*PU.*) and bounded peripherally by the feather sheath (*SH.*). The barb (*B.*) lies at the apex, and

[3] It will be useful to consider each barb and its barbules as constituting a miniature vane in the sense of Pernitza (1871), which will henceforth be referred to collectively as the "barb-vane" of Jones (1907).

one row of barbules (*B'BL. PL.*) occupies each lateral margin of each ridge. Since all barb-vanes are folded up in this fashion within the feather sheath, the entire circumference of the feather germ is divided into as many longitudinal ridges as there will be barb-vanes in the completed feather. This breaking-up of the walls of the feather germ into

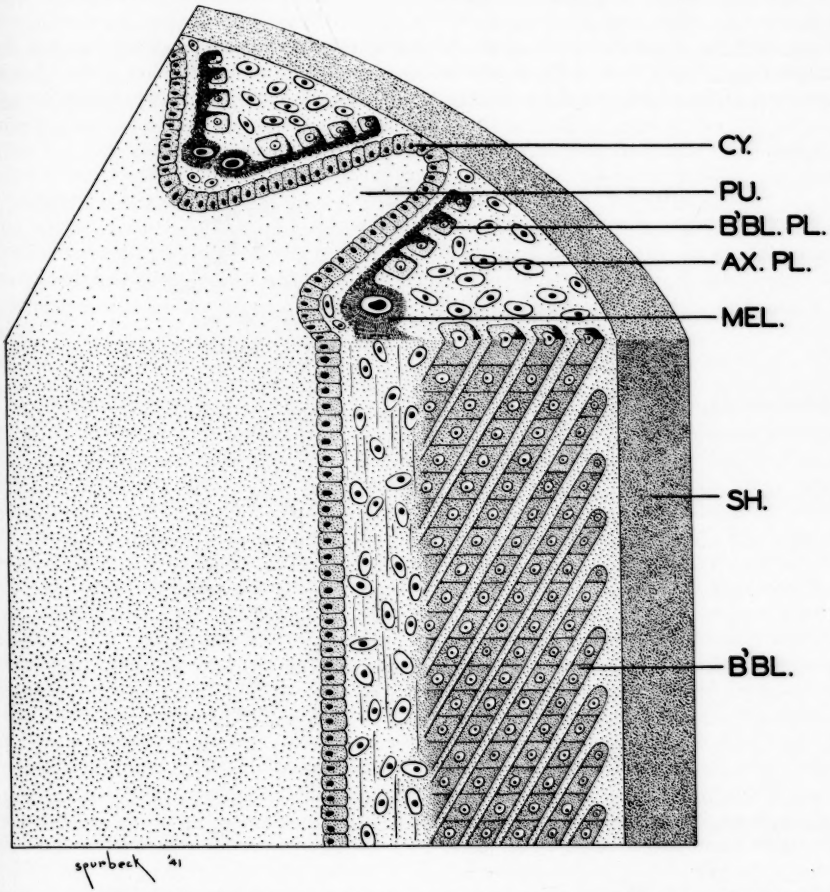


FIG. 3.—The deposition of pigment into barbule cells of two barb-vane ridges of an 11-day feather germ (diagrammatic). *AX. PL.*=axial plate; *MEL.*=melanophore. Other labels as before.

longitudinal ridges results in the change from a random distribution of pigment cells to a definite arrangement into several rows, each corresponding to one ridge.

An even earlier stage in the differentiation of two ridges, characteristic of an 11-day feather germ, represents the one time during development that barbule cells can receive pigment (text Fig. 3). Barbs have not yet formed, but the cells from which they will arise are those lying between barbule plates and are spoken of collectively as the "axial

plate" (*AX. PL.*) of each ridge (Strong, 1902). The cell bodies of melanophores (*MEL.*) now occupy the pulp margin of each ridge (future site of barbs). Their long processes extend peripherally, usually between cylinder cells and barbule plates, and carry melanin granules to barbule cells.

### 7. RIDGE FORMATION

At first the epidermal walls of the feather germ constitute a smooth cylinder. Two longitudinal ridges then arise *in situ* on the dorsal side of the feather germ (Jeffries, 1883). Additional ridges quickly appear to the right and left in dorsoventral order until

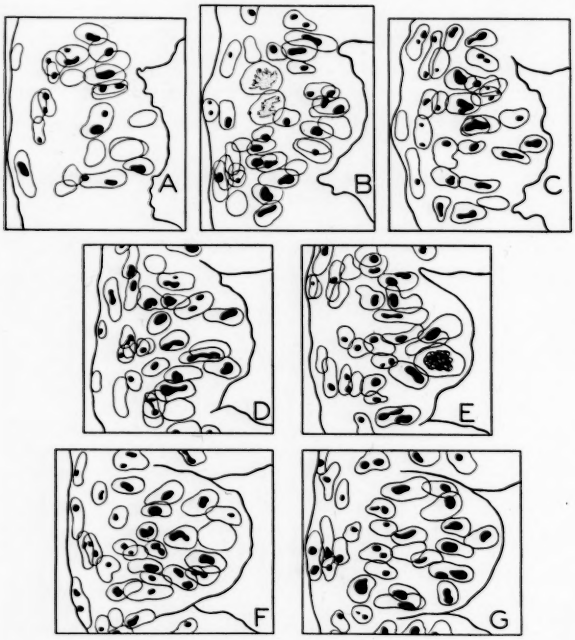


FIG. 4.—*Camera lucida* drawings of successive basoapical transverse sections through one barb-vane ridge developing in a feather germ from a 10¼-day embryo. In each drawing the feather sheath is to the left, the epidermal-pulp boundary to the right.

the entire circumference of the distal third of the feather germ is divided into ten or eleven longitudinal ridges. These apical segments of barb-vane ridges are added to basally as the feather germ increases in length. The way in which the original ridges arise and the method whereby they elongate by incorporation of cells into their bases become evident upon examination of *camera lucida* drawings of successive basoapical transverse sections through one barb-vane ridge developing in a feather germ from a 10¼-day embryo (text Fig. 4). The epidermis progressively thickens somewhat during the formation of a ridge, but there is almost no increase in the number of epidermal cells

involved. The most obvious change is an increase in the volume of individual cells. This is indicated by the lengthening of the nuclei and particularly by the increased distance between them as ridge formation nears completion (cf. text Fig. 4, *B* and *G*). This is especially true of cells adjacent to the pulp, which thus assume their typical cylindrical shape. Because of the pressure of these growing and rearranging epidermal cells against one another and against the feather sheath, the dermoepidermal boundary becomes very irregular. At the completion of the process the inner layer of cylinder cells is quite readily distinguishable from the rounded intermediate cells inclosed by it. All structures of the down feather arise from intermediate cells. The outer flattened cells are sheath cells.

Within these newly formed ridges melanophores are still distributed at random. Their cell bodies may lie at the pulp margin of the ridge with their processes extending peripherally, or they may be located peripherally with their processes extending mesially. Only rarely is a melanophore present among the sheath cells (cf. Kawamura, 1933). Here cell processes never develop extensively, and melanophores fail to deposit pigment outside the cell body. During the rearrangement of the intermediate cells of each ridge into two lateral barbule plates and a central axial plate the melanophore cell bodies become aggregated at the inner side of each ridge. Their processes extend peripherally, coming into close association with peripheral barbule cells.

### 8. PIGMENT DEPOSITION

As early as the tenth day of incubation melanin granules begin to accumulate at the tips of melanophore processes. The tips become enlarged and bulbous and are pinched off, and blebs of pigment released in this way come to lie among the developing sheath cells (cf. Hamilton, 1940). During the rearrangement of intermediate cells bleb formation ceases temporarily, only to begin again when the processes of melanophores come into close association with barbule cells. Pigment granules contained in blebs are released by their breakdown and are taken up by the barbule cells. However, somewhat later the formation of blebs becomes less evident, and ultimately a much finer relationship is established between melanophore processes and barbule cells, whereupon there is a steady "flow" of pigment from the former into the latter without any visible break in the continuity of pigment contained in them (see Strong, 1902). Pigment deposition begins with the most peripheral barbule cells (cf. Post, 1894; Strong, 1902; Greite, 1931 and 1934; Kawamura, 1934; von der Eldern, 1936). As keratinization overtakes these cells, the melanophore processes withdraw progressively toward the pulp (see text Fig. 3), depositing pigment successively into more mesial cells. After barbule cells have received their pigment, the same melanophores distribute it to the prospective barb cells of the axial plates. The structural elements of the down feather can receive pigment only at this stage of their development and only from such branched melanophores.

The striking picture which is presented by melanophores at the height of pigment deposition is best observed from the pulp boundary of each ridge (Pl. III, Fig. 18). The cell bodies are arranged in a row at the pulp margin of each ridge. Their branched processes extend peripherally on each side, transporting pigment to barbule cells. Melanophores of one ridge may deposit melanin granules in cells of an adjacent ridge, although this occurs only exceptionally (see Kawamura, 1933). In such cases a melanophore process may penetrate the two cylinder cell layers separating the ridges (see text Fig. 3), but more frequently it extends peripherally along the cylinder cell layer of one ridge and passes over into the next ridge through the layers of inner sheath cells.

### 9. FORMATION OF ADDITIONAL RIDGES

Although the completed down feather consists of from ten to fifteen barb-vanes, only ten or eleven barb-vane ridges arise in dorsoventral order around the circumference of the feather germ. Additional ridges arise in one of two ways. As the feather germ increases in diameter, the ridges may fail to increase correspondingly, and a triangular-shaped space may appear between the bases of two ventral ridges. The tip of a new ridge begins to develop here, and it pushes adjacent ridges apart until it can occupy its definitive position in the circle of barb-vane ridges. If a second ridge arises in this way, it is never adjacent to the one most recently formed but is separated from it by at least one of the original complement of ridges.

Additional ridges may also arise by the splitting of one which is already present. Normally pigment is not deposited into axial plate cells until all barbule cells at that level of the feather germ contain pigment. However, in a ridge which is destined to become split, pigment is deposited into a group of cells lying in the axial plate region (Pl. III, Fig. 19). These pigmented cells quickly become divided into two groups (Pl. III, Fig. 20), each of which contains three or four rectangular cells arranged in a plate which lies roughly parallel to one of the two barbule plates. The cells between the new plates differentiate as cylinder cells. Additional pigmented cells are added to the mesial end of each new plate, and the cylinder cell layers between them also extend mesially. The pulp margin of the ridge now undergoes an indentation at its center, whereupon the cylinder cells adjacent to the pulp become continuous with those separating the two new barbule plates (Pl. III, Fig. 21). Two barb-vane ridges now exist where only one was present before. As a result of this splitting process, cells which originated in the axial plate region of one ridge have been distributed to two ridges and will develop into barbules attached to two different barbs.

Since normally one barb-vane ridge produces one barb-vane and since the barb-vane ridge does not split throughout its entire length, the two barb-vanes arising from these new ridges should be fused apically throughout part of their length, and this is actually the case (Pl. III, Fig. 22).[4] Splitting always occurs below the barbule-free apex of the barb-vane. It may begin at any point between here and the base of the down feather. The maximum number of split barbs per feather appears to be three. Occasionally one ridge divides into two; and one of these, in turn, splits into two more.

Within one feather germ, ridges, in addition to the first complement, may arise by the formation of a new ridge between the bases of two of the original complement or by the splitting of one of the original ridges or by both methods.

### 10. THE DISTRIBUTION OF MELANOPHORES UNDERGOING MITOSIS

Definitive melanophores frequently undergo typical mitotic division. Before feather-germ formation begins, dividing melanophores are uniformly distributed throughout wing-bud epidermis. During early stages of feather-germ development melanophore pro-

[4] These split barbs are identical with the "united barbs" which Lillie and Wang (1941) have produced experimentally in regenerating feathers. They suggest that such a condition arises when the organization center of a single barb divides and the two centers then grow independently. They believe that the stimulus for this division is due to faulty tangential movement following operative procedures. In down feathers from the wings of Barred Rock chicks this union, or splitting, is a rather common occurrence. At least one split barb was present in eleven out of thirty wing feathers taken from nine birds. These appeared in the absence of other abnormalities. Perhaps their frequent occurrence may be associated with the complete absence of tangential movement in developing down feathers.

liferation becomes restricted to these localized epidermal thickenings along the outer margin of the wing. From the onset of feather development until pigment deposition begins, dividing melanophores are uniformly distributed throughout the intermediate cell layers of the feather germ. However, with the onset of melanin deposition melanophore proliferation becomes definitely localized at those levels of feather germs where active deposition is in progress (text Fig. 5). As the region of pigment deposition moves basally during older stages, the locus of melanophore proliferation also shifts in this direction.

Post (1894) and Kawamura (1933) have accurately described mitotic figures in bird melanophores. During the prophase the branched processes are usually withdrawn, and the cell body becomes enlarged and rounded. The spindle frequently appears much looser than that of adjacent epidermal cells (cf. Champy, 1935). In the epidermis of feather germs division of the cytoplasm usually coincides with the nuclear telophase, whereas in tissue culture it may be retarded, resulting in the formation of chains of melanophores (see Hamilton, 1940).

FIG. 5.—The basoapical distribution of melanophores undergoing mitotic division in an 11- and 12-day feather germ. Each dot indicates one melanophore in some stage of mitosis. Reconstructed from serial sections.

### 11. DERMAL MELANOPHORES

Pigment cells may or may not be present in the pulp of developing down feathers. They first appear at about $10\frac{1}{4}$ days of incubation, and they reach their maximum development by 11 days (Pl. III, Fig. 23). They may be present as single bipolar cells or as long chains consisting of several cells joined end to end. In either case they usually extend lengthwise of the dermal papilla, although occasionally they lie transverse to the long axis of the feather germs. They may be concentrated near the center of the pulp, but they are frequently crowded against the cylinder cell layer of the epidermis. However, they have never been observed to penetrate this layer, and there is no other indication that a migration of pigment cells ever occurs from the pulp into the epidermis of developing down feathers (contrast Champy, 1935). Neither is there any histological evidence which would suggest that melanoblasts ever enter the epidermis after the development of the down feather begins.

### 12. COMPLETION OF PIGMENT DEPOSITION AND FATE OF MELANOPHORES

The orderly arrangement of melanophore processes transverse to the length of barb-vane ridges disappears during the completion of barbule pigmentation and the onset of distribution of pigment to prospective barb cells of the axial plates. Melanophore processes become extended longitudinally along the pulp margin of each ridge (Pl. IV, Fig. 24). During barb formation melanophores quickly become reduced to dense balls of pigment, each surrounding a degenerating nucleus (Pl. IV, Fig. 25). These become crowded toward the pulp, where they are inclosed by cylinder cells and by any residual epidermal cells which failed to become incorporated into barbs or barbules. The pulp begins to dry rapidly and to shrink; and, as it shrinks, it pulls the cylinder and residual cells (and any

inclosed melanophores) away from keratinizing barbs and barbules (see text Fig. 2). This central mass is quickly reduced to a heavily pigmented, flattened, ribbon-like strand of cells (Pl. IV, Fig. 26), which extends throughout the entire length of the feather germ, attached only to the calamus. After hatching, when the feather sheath is lost and the barb-vanes assume their definitive positions, this cell strand is present temporarily in the center of each down feather. However, it is quickly broken off and lost, carrying with it the remains of the melanophores.

In the distal third of each barb-vane ridge, which gives rise to the barbule-free extremities of the barbs, there is no differentiation into barbule plates and there is no tendency for melanophore cell bodies to become shifted toward the pulp margin of the ridges. When keratinization of the surrounding cells occurs, these melanophores are trapped by the barb cells and become incorporated into the barbs (see Hosker, 1936). Such a fate of melanophores is peculiar to the distal regions of barb-vane ridges.

### 13. PIGMENTATION OF THE CALAMUS

The structure of the calamus is exceedingly variable in the down feathers of flight feathers. According to Davies (1889), the calamus is typically a cylinder within a cylinder. The inner cylinder is formed by the fusion of the bases of the barbs; the outer one consists of sheath cells, which at this level of the down feather always adhere to the intermediate cells and are not lost after hatching, as is the feather sheath. The calamus breaks up distally into the barbs of the down feather and proximally into those of the definitive feather. Such a typical calamus is formed occasionally in Barred Rock wings, but usually the inner cylinder is considerably modified. There may be no fusion of barb bases at all, such that each barb of the down feather can be traced through the level of the calamus until it breaks up into two or three barbs of the juvenile feather. This is the condition which Jones (1907) regards as typical, and this is the usual situation in the primary flight feathers. But there may be all transitions between these two extremes, i.e., there may be two barb bases fused together or any number up to the entire complement.

The calamus always contains much less pigment than either the barb-vanes of the down feather or the tips of the juvenile feathers, as Post (1894) has observed. Accordingly, the junction between these two feathers is sharply marked by a light-colored region (Pl. II, Fig. 16). If the inner cylinder of the calamus is formed by complete fusion of barb bases, almost no pigment is deposited, and rounded clumps of pigment (degenerate melanophores) are scattered among the epidermal cells. However, if the barbs maintain their identity throughout this region, considerable pigment is deposited, and degenerate melanophores packed with pigment are much less common. The transition from the heavily pigmented barb-vanes of down feathers to the lightly pigmented calamus is always gradual, whereas the change from the lightly pigmented calamus to the heavily pigmented tips of juvenile feathers is very abrupt.

### 14. GROWTH OF DOWN AND JUVENILE FEATHERS DURING INCUBATION

There is considerable variation from embryo to embryo in the length of a particular down feather at any one stage of incubation. In order to evaluate the extent of this variation and in order to obtain the growth curves for down and juvenile feathers during incubation, the following procedure was used. The right wing was removed from at least seven Barred Rock embryos of each sex at each stage of development. The wings were placed in saline, and the feathers were dissected out of secondary follicle No. 2. The

distance from the tip of the feather sheath to the base of the dermal papilla was measured in micrometer units, and the mean length (*M* length) and standard error (S.E.) were calculated for each stage (Table 1). From the seventeenth to the twenty-first days of

TABLE 1

GROWTH IN LENGTH OF FEATHERS PRODUCED DURING INCUBATION BY SECONDARY FOLLICLE NO. 2

| Age in Days | Sex | Down+Juvenile Feathers | | Juvenile Feathers | |
|---|---|---|---|---|---|
| | | No. of Feathers Measured | *M* Length±S.E.* | No. of Feathers Measured | *M* Length±S.E.* |
| 9½ | ♂ | 8 | 5.13±0.87 | ..... | ..... |
| | ♀ | 16 | 4.25±0.68 | ..... | ..... |
| 9¾ | ♂ | 9 | 5.11±0.87 | ..... | ..... |
| | ♀ | 10 | 8.10±0.56 | ..... | ..... |
| 10 | ♂ | 8 | 9.90±0.95 | ..... | ..... |
| | ♀ | 10 | 9.80±0.84 | ..... | ..... |
| 10¼ | ♂ | 11 | 14.00±0.50 | ..... | ..... |
| | ♀ | 12 | 13.58±0.39 | ..... | ..... |
| 10½ | ♂ | 10 | 16.00±1.38 | ..... | ..... |
| | ♀ | 9 | 13.78±1.36 | ..... | ..... |
| 10¾ | ♂ | 8 | 20.12±1.26 | ..... | ..... |
| | ♀ | 9 | 20.22±1.32 | ..... | ..... |
| 11 | ♂ | 14 | 35.78±1.44 | ..... | ..... |
| | ♀ | 7 | 36.86±1.79 | ..... | ..... |
| 12 | ♂ | 12 | 97.67±4.33 | ..... | ..... |
| | ♀ | 11 | 100.91±5.29 | ..... | ..... |
| 13 | ♂ | 8 | 208.13±5.29 | ..... | ..... |
| | ♀ | 8 | 195.13±6.94 | ..... | ..... |
| 14 | ♂ | 12 | 209.33±9.16 | ..... | ..... |
| | ♀ | 9 | 222.44±6.56 | ..... | ..... |
| 15 | ♂ | 7 | 229.86±6.73 | ..... | ..... |
| | ♀ | 12 | 246.92±7.28 | ..... | ..... |
| 16 | ♂ | 28 | 251.75±7.14 | ..... | ..... |
| | ♀ | 15 | 253.87±7.99 | ..... | ..... |
| 17 | ♂ | 19 | 270.21±7.11 | 8 | 26.38±1.90 |
| | ♀ | 23 | 273.74±5.31 | 13 | 24.54±2.04 |
| 18 | ♂ | 20 | 272.80±5.18 | 8 | 32.50±2.34 |
| | ♀ | 22 | 266.09±5.37 | 8 | 39.50±2.31 |
| 19 | ♂ | 22 | 281.32±6.89 | 10 | 54.20±3.45 |
| | ♀ | 19 | 302.21±8.98 | 13 | 67.92±4.11 |
| 20 | ♂ | 20 | 323.70±6.27 | 20 | 79.70±5.43 |
| | ♀ | 21 | 321.33±4.84 | 21 | 77.00±3.01 |
| 21 | ♂ | ..... | ..... | 19 | 86.79±6.26 |
| | ♀ | ..... | ..... | 25 | 90.36±5.09 |

* Data are given in micrometer units (19.25 units = 1 mm.).

incubation the lengths of the juvenile feathers alone are also recorded. The growth curves are plotted separately for males and females in text Figure 6.

The feather germ slowly increases in length until ridge formation involves the upper two-thirds of the feather germ, whereupon the growth rate increases abruptly at 10¾

days. Between $10\frac{3}{4}$ and 13 days the down feather completes its growth. The most rapid elongation occurs between the twelfth and thirteenth days, during which time the average increase in length is approximately 5.5 mm. This is an exceedingly high rate of growth, even when compared to the rapidly elongating juvenile feathers of White Leghorns (Willier and Rawles, unpublished data). Thereafter growth decreases abruptly;

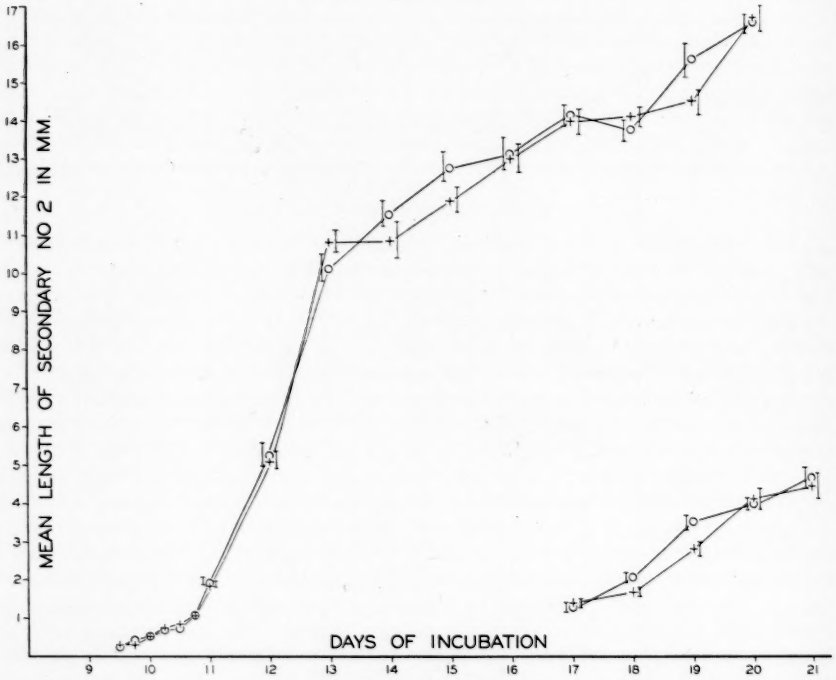


FIG. 6.—The curves of growth of male and female feathers produced by secondary follicle No. 2 during incubation (+ = males, ○ = females). From $9\frac{1}{2}$ to 13 days the curves represent the growth of the down feather. Beyond 13 days the upper curves record the combined length of down and juvenile feathers. The shorter curves plotted from the seventeenth day until hatching indicate how much of this total growth is due to elongation of juvenile feathers. The upper and lower limits of the vertical bar to the right of each pair of points are, respectively, *M* length *plus* S.E. and *M* length *minus* S.E. for males. Those to the left are, respectively, *M* length *plus* S.E. and *M* length *minus* S.E. for females.

and, if the data are plotted logarithmically, it becomes evident that there is a definite cessation in growth between the completion of the down feather and the onset of juvenile feather formation. These observations are contrary to those of Jones (1907), who stated that "the first down and its succeeding definitive feather are produced by one continuous growth, and therefore cannot be regarded as two distinct feathers" (p. 17).

It will be noted that between the seventeenth and eighteenth days, although the juvenile feathers have increased in length, the total length (down + juvenile) remains

relatively constant in males and actually decreases in females. This irregularity is due to the fact that at this time the pulp has contracted and the barb-vanes of the down feathers have become free within the sheath, so that now the feather sheath is flattened, instead of cylindrical. This rearrangement brings about a shortening and a broadening of the feather sheath. The lengths of male and female feathers differ statistically only at $9\frac{3}{4}$ and 19 days of incubation.

## IV. DISCUSSION

### 1. MELANOPHORE MIGRATION

The source and function of the stellate pigment cells which Remak (1855) first observed within the skin and feather germs of chick embryos have long been problems of considerable interest. His observations on the location of these cells and their gross rearrangements during the morphogenesis of down feathers were extremely accurate. Nevertheless, it was Klee (1886), Kölliker (1887), and Haecker (1890) who first recognized the true significance of these melanophores, viz., that they are the source of the melanin granules contained in the epidermal constituents of feathers of pigmented breeds. However, Post (1894) and Strong (1902) were the first to describe melanin deposition in any detail.

Attempts to ascertain the source of these melanophores were immediately forthcoming, and from 1886 until 1936 there were numerous histological investigations stimulated by this problem. Klee (1886) concluded that melanophores of chick embryos originate in the dermis, whence they migrate into the epidermis and deposit their pigment. In addition, Klee attributed to epidermal cells the ability to form pigment *in situ*. Kölliker (1887) confirmed Klee's first observation, and more recent studies on regenerating feathers also led Champy (1935) to emphasize the dermis as the source of melanophores.

However, Jeffries (1883), Rabl (1894), Hosker (1936), and von der Eldern (1936), working with chick embryos, and Kawamura (1934), studying quail embryos, were equally emphatic in stating that melanophores are epidermal cells. Their conclusion was supported by studies on regenerating feathers pursued by Post (1894), Strong (1902, 1915, and 1917), Lloyd-Jones (1915), Greite (1934), and Portmann and Gerber (1935).

Obviously, the source of melanophores cannot be decided by histological methods alone. The only criterion used in early studies to distinguish a melanophore from other cell types was its melanin content. Accordingly, the developmental history of these cells prior to melanin synthesis was unknown. More recently Kawamura (1934) was able to follow their early history somewhat farther by differential staining with osmic acid. The cytoplasm of potential melanophores was blackened, thus enabling him to trace the melanophores back to $5\frac{1}{2}$-day embryos. He believed he could recognize melanoblasts by their characteristic branched form even in 4-day embryos. But eventually he was forced to state: "Ich konnte aber keinen Beweis dafür liefern, dass meine sogenannten epidermalen Melanophoren mesodermale Elemente seien" (p. 95). However, Biedermann (1928) warned that exponents of the epidermal origin of melanophores should not overlook the possibility that mesenchymatous elements wandered into the epidermal layer at even earlier stages. Biedermann supported the interpretation of an extrinsic origin of melanophores particularly on the basis of an observation of Kerbert (1876) that during the development of scales on the legs of chick embryos cells filled with pigment lie partly in the dermis, whereas their branched processes penetrate into the epidermis and carry melanin granules to epidermal cells.

That melanophores of the epidermis do have an extrinsic origin and do migrate into it much earlier than previously anticipated is evident from the observations recorded in this paper. Between the eightieth and the ninety-first hours of incubation cells interpreted as precursors of melanophores migrate into the epidermis of wing buds. Several days after they have invaded this layer, melanoblasts begin the formation of melanin and thus become definitive melanophores. These are uniformly distributed in the epidermis of the outer margin of the ulnoradial level of the wing several hours before the onset of feather-germ formation. This means that at this level of the wing the epidermis which becomes incorporated into a down feather already contains melanophores before feather-germ formation begins. Melanophores which happen to lie in the epidermis between the bases of feather germs quickly undergo degeneration and disappear, as Kawamura (1934) has already observed in the dorsal epidermis of quail embryos. They may be present in the dermal pulp, but there is no histological evidence that either they or melanoblasts ever migrate from the pulp into the epidermis during down-feather development. It would appear that the pigment cells involved in the pigmentation of any down feather arise only by mitotic division from those few melanoblasts which happened to invade its epidermis before the onset of its development.

Some of the down feathers of Barred Rock chicks are nonpigmented. These white feathers are characteristically present on the wing tips and the undersurface of the body. Does this mean that melanoblasts failed to migrate into the epidermis in these regions? Several observations suggest that, although many melanoblasts enter the epidermis at the ulnoradial level of the wing, progressively fewer enter in strict proximodistal direction. (1) The white areas do not pass abruptly into surrounding heavily pigmented regions. Instead, there is a gradual transition, marked by the presence of (*a*) feathers which are lightly pigmented throughout, suggesting that only a few melanophores were involved in their pigmentation, i.e., that a reduced number of melanoblasts had entered the epidermis in such cases, and (*b*) mosaic feathers composed of pigmented and unpigmented barb-vanes. Almost without exception the pigmented barb-vanes are on the side of the feather nearest the source of melanophores, suggesting that only the proximal portion of the epidermis of these feather germs had been invaded by melanoblasts. (2) Pigmented feathers are never interspersed among the completely white feathers.

That the absence of melanophores in the white-feathered wing tips is not due to some peculiarity of the epidermis of feather germs in these regions is evident from the fact that black down feathers do occasionally arise here. The size of the white areas varies considerably among chicks and even between the two wings of the same bird. There may be as many as twenty white feathers on each wing tip, or all of them may be pigmented. This latter observation indicates that potential pigment cells can migrate into the epidermis of wing tips and that, when present, they differentiate normally and deposit pigment. However, if they do not reach the wing tip before the onset of feather development, they are apparently blocked from entering the epidermis throughout the development of down feathers.

The question next arises as to the nature of the block to cell migration imposed during feather-germ development. That such a block must exist is evident from Plate IV, Figure 27. It will be seen that pigment cells have ultimately reached the wing tip, since the dermis surrounding the follicles which contain the bases of the white down feathers is filled with melanophores, and yet they fail to enter the epidermis of the down feathers. Whatever the nature of the block, it is apparently removed suddenly, since, when the

organization of the tips of the juvenile feathers which succeed the white down feathers takes place (*arrow*), melanophores make an abrupt appearance in the epidermis (Pl. IV, Fig. 27). The transplantation experiments of Danforth (1937 and 1939) and Rawles (unpublished) support the hypothesis that a block to cell migration is lacking in juvenile feather germs. Danforth's skin transplants between newly hatched chicks of different breeds occasionally develop mosaic juvenile feathers at their margins. The follicles which produce such feathers contain both donor and host melanophores, indicating that host melanophores have migrated into juvenile feather germs. When areas of wing skin and feather germs, kept free from pigment cells by implantation into the coelom, are transplanted to the backs of newly hatched chicks of pigmented breeds, the juvenile feathers produced from the graft usually exhibit host pigmentation, demonstrating that melanophores readily migrate into juvenile feather germs (Rawles). These experiments artificially produce a situation quite comparable to that which normally occurs at the wing tips of Barred Rocks.

A comparison of the structure of the dermoepidermal boundary in down and regenerating feather germs may possibly suggest why a block to cell migration occurs in down feathers but is lacking in juvenile feathers. The epidermis of down feathers is sharply bounded from the dermis, whereas the dermoepidermal boundary of regenerating feathers is very poorly defined during early stages and in the ramogenous zone, where it is characterized by an interlacing of dermal and epidermal cells (Lillie and Juhn, 1938). If this boundary proves to be equally as indefinite in developing juvenile feathers, it would appear that the block to melanoblast migration in down feathers is mechanical in nature, perhaps similar to that which Ris (1941) described at the umbilicus of black chicks. The possibility of a physiological barrier to cell migration also exists.

A related problem is the transitory appearance of melanophore-free areas in the epidermis during the earliest stages of feather-germ formation. At first melanophores are evenly distributed. They begin to disappear from localized areas. Each melanophore-free area later becomes the side of a feather germ and occupies from one-fourth to one-third of its total surface. The boundaries of each spot become sharply defined and form a smooth curve on each feather germ (Pl. I, Fig. 11). There is no indication of degenerating melanophores. Apparently they either migrate out of these areas or are carried away by morphogenetic changes within the epidermis. The latter explanation appears to be more likely for several reasons. (1) In each feather germ the epidermis which contains no melanophores is thinner than that which contains many. Later, when the melanophore-free spots disappear, the epidermis is of the same thickness on all sides of the feather germ. (2) In later developmental stages visible shiftings of definitive melanophores are definitely the result of morphogenetic movements, rather than due to any ability of the pigment cells to migrate. The spots form in relation to the body axes of the bird. On the wings they occupy the proximal side of each feather germ, whereas on the back and tail they lie on the anterior side.

### 2. SEXUAL DIFFERENCES IN MELANIN PRODUCTION

The female Barred Rock embryo is darker than the male almost from the first appearance of melanin granules. This difference reaches its maximum development on the tenth day of incubation, when the pigment is still contained within melanophores. Later, during the deposition of pigment, sexual differences are much less obvious, but they still exist. They become particularly evident during the pigmentation of the proximal third

of the barb-vanes. In males this region remains relatively free from pigment (Pl. IV, Fig. 28), whereas in females each barb-vane is heavily pigmented throughout its length (Pl. IV, Fig. 29). As a result of this sexual difference in the distribution of melanin the down plumage of females is jet black, whereas that of males has a grayish cast. The possibility of determining the sex of chicks by these differences is immediately suggested. Jerome (1939) and Quinn and Knox (1939) attempted to classify day-old Barred Rock chicks as males or females particularly on this basis, although they supplemented this criterion with observations on sexual differences in the character of the head spot and in pigmentation of shanks and feet. They can successfully determine sex in from 93 to 96 per cent of the birds.

It has long been known that the juvenile and adult plumage of female Barred Rocks is darker than that of males. The black bars are both wider and darker in females. Spillman (1909) suggested the classical genetic explanation of this sexual dimorphism. He assumed that the sex-linked barring factor interferes with melanin production. Since males are homogametic, they contain two barring factors, which inhibit melanin production to a greater extent than the single factor present in heterogametic females, and therefore males are lighter in color. Whether the interference affects melanin formation or melanin deposition was not considered. This factor acts independently of sex hormones. A purebred Barred Plymouth Rock pullet developed an ovarian tumor, whereupon the plumage became similar to that of males in shape and structure but retained the quality of barring characteristic of hen feathers of this breed (Cole and Lippincott, 1919). Neither castration nor injections of sex hormones modify the feather patterns (Montalenti, 1934). Moreover, male and female Barred Rock melanophores implanted into host feather germs of nonbarred breeds produce male and female barred patterns, respectively, regardless of the sex of the host (Willier, 1941). Therefore the barring factor must act directly within melanophores. Its primary effect must be on melanin formation rather than on deposition, since male and female feather germs are distinguishable, even before pigment deposition begins, by differences in the amount of pigment contained in melanophores of the two sexes.

The possibility exists that differences in the amount of pigment deposited in the proximal third of male and female down feathers can be correlated with differences in the growth rate of feathers during the formation of these regions. Many investigations have demonstrated that the growth rate of a feather is related to the intensity of melanin deposition in such a way that rapidly growing feathers tend to become more heavily pigmented than slowly growing ones. Juhn (1937) studied the growth rates and coloration of three feathers successively produced by a single tail follicle of a Brown Leghorn. Each feather grew more rapidly and contained more black than its predecessor. Similarly, slowly growing juvenile and adult feathers of Barred Rocks have wider white bars (i.e., less melanin) than more rapidly growing ones (Montalenti, 1934). Conversely, feathers from rapid-feathering birds tend to exhibit less distinct white bars (i.e., more pigment) than those from slow-feathering birds (Martin, 1929). Moreover, Barred Rock melanophores functioning in slowly growing New Hampshire Red feather germs deposit less black pigment than those developing in rapidly growing White Leghorn germs (Willier, 1941).

However, a comparison of the growth curves of male and female down feathers clearly demonstrates that there is no statistical difference in the growth rate except at $9\frac{3}{4}$ days, which is almost a day before pigment deposition begins. Perhaps during incubation there

are physiological differences between male and female feather germs other than in growth rate, and these differences may be responsible for the different distribution of pigment in the two sexes. But the early cessation of pigment formation and deposition in male feather germs may be only the extreme effect of the presence of two barring factors, in the melanophores, which always appear to exert a greater inhibitory effect on pigment production than does a single factor.

### 3. DEVELOPMENTAL MECHANICS OF RIDGE FORMATION

One of the interesting problems of feather development is the nature of the mechanics involved in dividing the epidermal cylinder of the feather germ into the apical segments of barb-vane ridges and in the extension of these ridges basally. Various explanations have been proposed. Some have suggested that there are localized regions of epidermal cell proliferation around the circumference of the feather germ and that each such localized region produces one barb-vane ridge (Studer, 1873; Biedermann, 1928). However, mitoses are rare at those levels of the feather germ where ridges are rapidly elevated into the pulp, as Klee (1886) has already emphasized. It would appear more probable, therefore, that ridges are formed by the rearrangement of cells which are already present. Others believe that a pressure is built up between the growing pulp and the sheath, which causes the epidermal cells lying between these two regions to undergo rearrangement into ridges (Pernitza, 1871; Klee, 1886; Hoadley, 1926; Hosker, 1936). This pressure might be due to the rapid proliferation of dermal cells or to the development of a pulp turgor such as Lillie (1940) has demonstrated in regenerating feathers. However, such an explanation is improbable for several reasons: (1) Pulp cells are never forced between adjacent ridges, which might be expected if a considerable pressure were developed. (2) The formation of split ridges does not begin at the epidermal-pulp boundary, where a pulp pressure would be exerted, but in the distal part of the axial plate region. Therefore, the initiation of ridge formation, at least in this specialized case, appears to be independent of pulp pressure. (3) Finally, the irregularity of the pulp-epidermal boundary during ridge formation is of such a nature that it would suggest a piling-up of cells due to growth pressure within the epidermis and not to a pressure exerted on the epidermis from the pulp. It is the cylinder cells which undergo the greatest increase in size, particularly in width. The intermediate cells are also enlarging to a lesser extent. The outer layers of sheath cells constitute a relatively rigid structure, so that the growth pressure is effective only in forcing epidermal cells toward the less rigid pulp. The final shaping of the ridge appears to result from the pressure of the two adjacent ridges, whose cells are likewise undergoing rapid increase in size. There is no indication of a temporary appearance of an axial column of cells around which each ridge is formed, such as Greite (1934) has described in regenerating feathers.

Lillie and Juhn (1938) regard the basal growth of barb-vane ridges of regenerating feather germs as due to "their own intrinsic utilization of material of the ramogenous zone derived from the collar." The collar is a ring of embryonic cells which surrounds the base of the pulp of the feather germ. The ramogenous zone is located in the angle formed by the collar and the vertical walls of the feather germ. They believe that the apical segments of barb-vane ridges act as organizers of ramogenous material and incorporate these cells into their bases. Lillie and Wang (1941) postulate in each barb-vane ridge a center of undifferentiated cells in juxtaposition with the collar and designate this region as the "organization center." In the down-feather germ the collar and ramogenous zones

are not so distinct morphologically, but their functional existence is evident. The barb-vane ridges of down-feather germs increase in length by the aggregation of cells at their bases rather than as a result of localized regions of cell divisions corresponding to their bases.

It is understandable how the melanophores become mechanically grouped into one or another of the apical segments of developing ridges during their formation. However, it is somewhat more difficult to see how during ridge formation the cell bodies of melanophores become shifted toward the pulp margin of the ridges. Those melanophores which lie nearer the pulp from the beginning of ridge formation appear to divide more rapidly than those which are located nearer the sheath. This would account for the greater number of melanophore cell bodies concentrated at the pulp margin, but it still would not explain their complete disappearance from the outer surface of each ridge. This latter phenomenon is associated with the formation of barbule plates, since in the distal third of feather germs, where no barbule plates arise, no aggregation of melanophores at the pulp margins of ridges occurs.

### 4. BILATERAL ORGANIZATION OF DOWN-FEATHER GERMS

The fact that the first complement of barb-vane ridges arises in strict dorsoventral order demonstrates that the down feather, although apparently a radially symmetrical structure when completed, possesses an underlying bilateral organization during its formation, as has been suggested by Wohlauer (1902), Jones (1907), Schleidt (1913), Lamont (1922), and von der Eldern (1936). It is therefore easier to understand how one feather follicle can produce in succession the radially symmetrical down and the bilaterally symmetrical juvenile feathers which differ so greatly in their definitive forms. In feather germs of regenerating feathers the embryonic area capable of producing additional ridges is restricted to the mid-ventral line (see Lillie and Juhn, 1932 and 1938). In those of down feathers, this area is more extensive, since additional ridges can arise between the bases of any two ridges of the first complement, although they usually arise in the ventral half of the feather germ. Additional ridges which arise by the splitting of the ridges formed in the first complement may arise equally as well in the dorsal as in the ventral half of the feather germ.

### 5. THE DEPOSITION OF PIGMENT

One of the questions which has interested investigators for a long time is the nature of the relationship between the pigment cell and the barbule cells which leads to pigment deposition. Previously the pigment cell has been considered to be almost a micro-injection apparatus capable of injecting pigment granules into passive recipient barbule cells. However, several lines of evidence suggest that a much more active role is played by barbule cells during the pigmentation process. (1) Pigment cells are loaded with pigment granules at an early stage, even before longitudinal ridges have formed. Nevertheless, pigment is not deposited in epidermal cells until certain of these become visibly differentiated in the direction of barbule cells, whereupon they begin to receive pigment. (2) Before any definite barbule cell differentiation occurs within a ridge, pigment granules accumulate at the tips of pigment cell processes and are pinched off and come to lie freely among the cells of the ridge. Pigment liberated in this manner is later taken up by barbule cells. (3) Pigment is deposited in each row of barbule cells in a definite sequence (see text Fig. 3). The melanophore process extends past the most centrally located bar-

bule cells and carries pigment first to the most peripheral cells and then progressively to more axial cells. This is the same order in which barbule cells undergo differentiation. The most peripheral barbule cells are the first to elongate and to form keratin; and only when these visible differentiation processes begin, can they receive pigment. As this wave of differentiation spreads progressively toward the pulp, the more axial cells in turn become capable of receiving pigment. (4) Pigment-cell processes appear to be specifically attracted toward barbule cells. Under normal conditions none are directed toward cells lying between barbule plates. However, occasionally a melanophore process does carry pigment to a group of cells lying in the axial plate region. This at first appears to be contradictory to the idea of a specific attraction; but, if the fate of these pigmented cells is followed, they are found to divide into two groups, and then the longitudinal ridge is divided apicobasally between them, whereupon cells which originated in the center of one barb-vane ridge become distributed between two ridges and are distinguishable as barbule cells in each ridge. These split barb-vane ridges develop into split barb-vanes. Thus, the development of these unusual down feathers clearly demonstrates that pigment-cell processes are specifically attracted to barbule cells at this time, even though they must leave their usual paths in order to reach them. (5) Pigment deposition stimulates the melanophores involved to undergo mitotic division. If the apicobasal distribution of pigment cells which are undergoing mitotic division is plotted from an 11- and a 12-day feather germ, the mitotic figures are definitely grouped within a relatively narrow region. It is only within this region that barbule cells are actively receiving pigment. (6) During the first day of its development the feather germ grows slowly; but, beginning suddenly at $10\frac{3}{4}$ days of incubation, the germ elongates rapidly, attaining its full growth by 13 days. The onset of pigment deposition coincides exactly with the onset of rapid growth. Lillie and Juhn (1932) have estimated that 90 per cent of the longitudinal growth of regenerating feathers is accomplished by cell elongation. It would appear that pigment deposition begins suddenly at that phase of development when barbule cells begin to elongate rapidly. (7) The down feather of Barred Rocks is solid black in color, whereas juvenile and adult feathers of this breed exhibit alternate black and white transverse bars. Willier (1941) has demonstrated that rapidly growing juvenile feathers of this breed produce a more nearly solid black pattern than more slowly growing ones. Its down feathers grow more rapidly than any of its juvenile feathers, elongating 5.5 mm. between the twelfth and thirteenth days. It is, indeed, a tempting thought that this rapid elongation stimulates continuous pigment deposition, so that a solid-colored down feather results.

Post (1894), Greite (1931 and 1934), and Champy (1935) have all suggested that barbule cells must attain a definite degree of differentiation before they can receive pigment. If this hypothesis is true, as is indicated by evidence presented here, it can explain the typical distribution of melanin-containing barbule cells at different levels of developing feather germs, although Greite (1934) does not follow this possibility to its logical conclusion.

The exact way in which epidermal cells receive pigment granules remains unknown. Hamilton (1941) has suggested, on the basis of his *in vitro* observations, the possibility that the barbule cells engulf the melanin granules by amoeboid movements; but Strong (1902) emphasized that in his fixed material the form of the barbule cells while they are receiving pigment is conspicuously uniform and constant, with no suggestion of phagocytic activity. Greite believes that pigment granules are deposited on barbule cells,

whereas Champy suggests that pigment-cell processes penetrate into barbule cells and carry pigment into their interior. All investigations, except those of Greite, agree that pigment granules are deposited inside of barbule cells.

### 6. FATE OF THE PULP AND DEGENERATE MELANOPHORES

The ultimate fate of melanophores is dependent upon that of the pulp. The studies of Davies (1889) and Lillie (1940) record in detail the resorption of the pulp and the accompanying pulp-cap formation in regenerating feathers. However, this process has not been accurately observed in the development of down feathers. Post (1894), Strong (1902), Greite (1931 and 1934), Champy (1935), and von der Eldern (1936) observed that degenerate melanophores become imbedded among the cylinder cells which lie adjacent to the pulp. Post believed that they migrate through the cylinder cell layer and come to lie among the pulp cells, but he did not follow the fate of the pulp. Von der Eldern merely stated that pigment cells are lost with the unfolding of the mature down feather. Both Post and Davies described the shrinkage of the pulp and the overlying cylinder cells away from the keratinized feather structures. According to Post, the cells and intercellular substance of the pulp are reduced. Davies stated that pulp tissue is very thin at this time and is apparently gradually absorbed. Actually, only the intercellular substance appears to be resorbed, whereas the pulp cells and blood cells remain inclosed by the cylinder cell layer and become cut off from the dermal papilla of the juvenile feather by the onset of pulp-cap formation in the calamus of the down feather. The resulting flattened strand of pulp and blood cells and overlying cylinder and pigment cells extends throughout the length of the feather germ and is broken off at the calamus and lost during, or shortly after, the release of the down feather from the feather sheath. The onset of pulp-cap formation must present a special modification of the process as described by Lillie (1940), since pulp tissue is cut off above and below this first cap. Its formation has not yet been studied histologically.

## V. SUMMARY

1. This paper is concerned with a histological study of the developmental history of melanophores in wing skin and feather germs of Barred Rock embryos from the onset of their migration into the epidermis until hatching. The morphogenesis of the down feather is considered only in so far as it is essential to an understanding of the behavior of melanophores.

2. The precursors of melanophores are first recognized at 80 hours' incubation, when their migration into wing-bud epidermis begins. They contain no pigment but are identified as melanoblasts because their first appearance coincides with the earliest stage in which Willier and Rawles (1940) have been able to obtain areas of donor-colored feathers by transplanting wing-bud epidermis. Migration continues from the eightieth to the ninety-first hour, when it ceases altogether.

3. Melanoblasts acquire pigment between 7 and 7½ days of incubation, i.e., several days after they have invaded the epidermis. They are evenly distributed at first. Melanophores persist and multiply only in those parts of the epidermis which become incorporated into feather germs.

4. Melanophores undergo successive changes in distribution as a result of morpho-

genetic movements within feather germs. (*a*) Accompanying the onset of feather-germ formation is a temporary appearance of a melanophore-free area on one side of each feather germ. Its location bears a definite relation to the axes of the body. (*b*) A change from a random distribution to an orderly arrangement of melanophores into longitudinal rows occurs when the epidermal wall of the feather germ is divided into barb-vane ridges. (*c*) The melanophores within ridges are progressively shifted toward the pulp as intermediate cells become arranged into axial and barbule plates.

5. Although pigment cells may occur in the dermal pulp, there is no evidence for their migration into the epidermis of developing down feathers.

6. Melanophore proliferation occurs at all levels of the down-feather germ prior to pigment deposition. Later their proliferation is restricted to those levels where active deposition is taking place.

7. Several lines of evidence suggest that barbule cells play an active role in pigment deposition. (*a*) Melanin contained in blebs pinched off by melanophores is taken up by barbule cells. (*b*) Epidermal cells can receive pigment only when they have reached a definite stage of differentiation, characterized by the onset of cell elongation and keratinization.

8. After pigment deposition ceases, degenerate melanophores become incorporated into the layer of cylinder cells which covers the desiccated pulp. This entire strand of cells is attached only at the calamus and is lost soon after hatching.

9. Evidence is presented which indicates that melanoblasts usually fail to reach the wing tip before 91 hours of incubation and are blocked from entering the epidermis thereafter. Therefore, no melanophores become incorporated into down feathers of the tips, with the result that the feathers are white. That this block to cell migration is possibly mechanical in nature is indicated.

10. Ridge formation results from a rearrangement of epidermal cells due to an increase in their volume and to the pressure which they exert against the sheath. It involves no localized regions of cell proliferation and is probably independent of pulp pressure.

11. The first complement of ridges arises in strict dorsoventral order, demonstrating the presence of an underlying bilateral organization in down feather germs.

12. Barb-vane ridges of down feathers frequently divide lengthwise in their proximal portions, giving rise to split barb-vanes which are comparable to the "united barbs" produced experimentally in regenerating feathers.

13. Female embryos have darker feather germs than males before pigment deposition into epidermal cells begins, because genetically female melanophores contain more pigment. The proximal third of the barb-vanes of males is lightly pigmented, whereas in females the barb-vanes are heavily pigmented throughout their length. This sexual difference in the distribution of pigment is independent of the growth rate of the feathers. It is probably genetically determined.

14. The growth in length of the down and juvenile feathers produced by secondary follicle No. 2 was measured throughout incubation. The down feather grows slowly from $9\frac{1}{2}$ to $10\frac{3}{4}$ days but rapidly from then until the thirteenth day. Between the twelfth and thirteenth days it grows approximately 5.5 mm. It is suggested that the melanophores respond to this rapid growth rate by functioning continuously and thus producing a nonbarred, uniformly black down feather.

15. The structure of the definitive down feather is described in detail.

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## PLATE I

FIG. 7.—A longitudinal section through the wing bud of an 82-hour embryo. Note melanoblast (*arrow*) entering epidermis (*above*) from dermis. ×1,000.

FIG. 8.—Whole mount of epidermis from the outer margin of the ulnoradial level of the wing of an 8-day embryo. Melanophores are uniformly distributed. ×210.

FIG. 9.—Whole mount of epidermis from the ulnoradial region of an $8\frac{3}{4}$-day embryo. Melanophores have disappeared from the proximal side of the feather germ, thus forming a melanophore-free spot. Contrast melanophore distribution with Fig. 8. ×100.

FIG. 10.—Whole mount of a 9-day feather germ. Compare size of individual melanophores with Fig. 9. ×100.

FIG. 11.—The tail of a 9-day embryo. The brief history of the melanophore-free areas can be followed in the two vertical rows of feather germs to the right of the center. ×26.

FIG. 13.—Ten-day male (*above*) and female embryos. Note difference in intensity of black in male and female feather germs of dorsal tracts. ×2.

## PLATE II

FIG. 12.—Whole mount of a feather germ from the wing of an embryo of $9\frac{7}{8}$ days' incubation. All the melanin is still contained within melanophores. Note random distribution of pigment cells. ×210.

FIG. 14.—The tip of a split feather germ from a $10\frac{1}{8}$-day embryo. Melanophores are undergoing an orderly arrangement into a series of longitudinal rows. Viewed from the pulp surface. Contrast melanophore distribution with Fig. 12. ×210.

FIG. 15.—Split feather germ from the wing of a $10\frac{3}{8}$-day embryo. All melanophores in the distal two-thirds are grouped into ten or eleven longitudinal rows. In the proximal third they still show a random distribution. Viewed from the pulp surface. ×100.

FIG. 16.—A cleared wing of a newly hatched chick showing the pin feathers of the juvenile flight feathers and the down feathers attached to their tips. ×2.5.

PLATE I

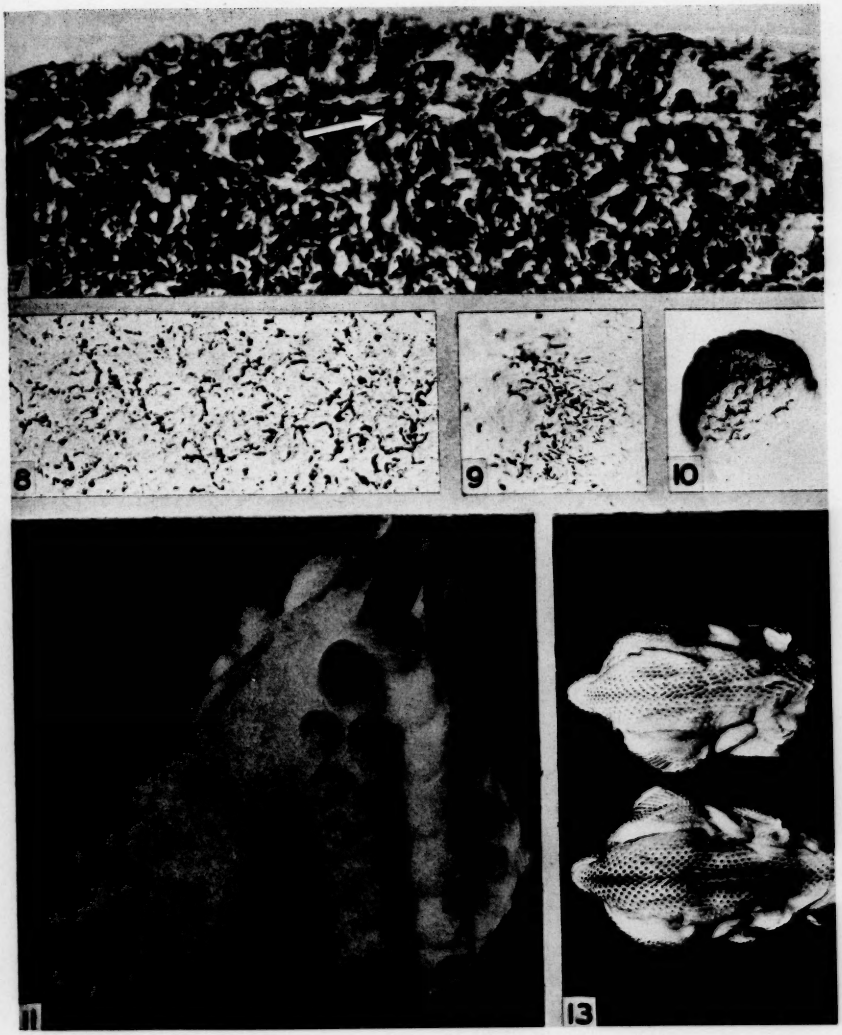

PLATE II

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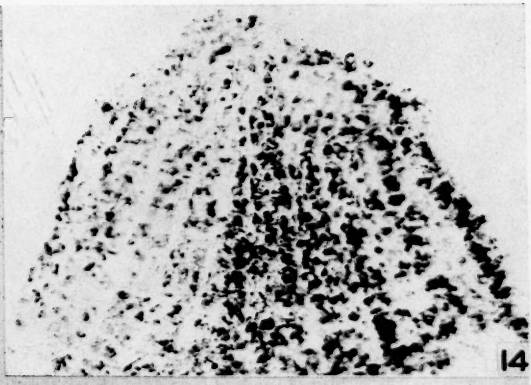
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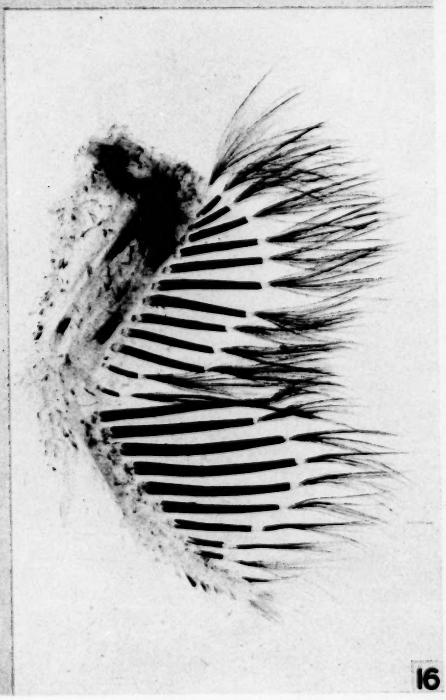
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PLATE III

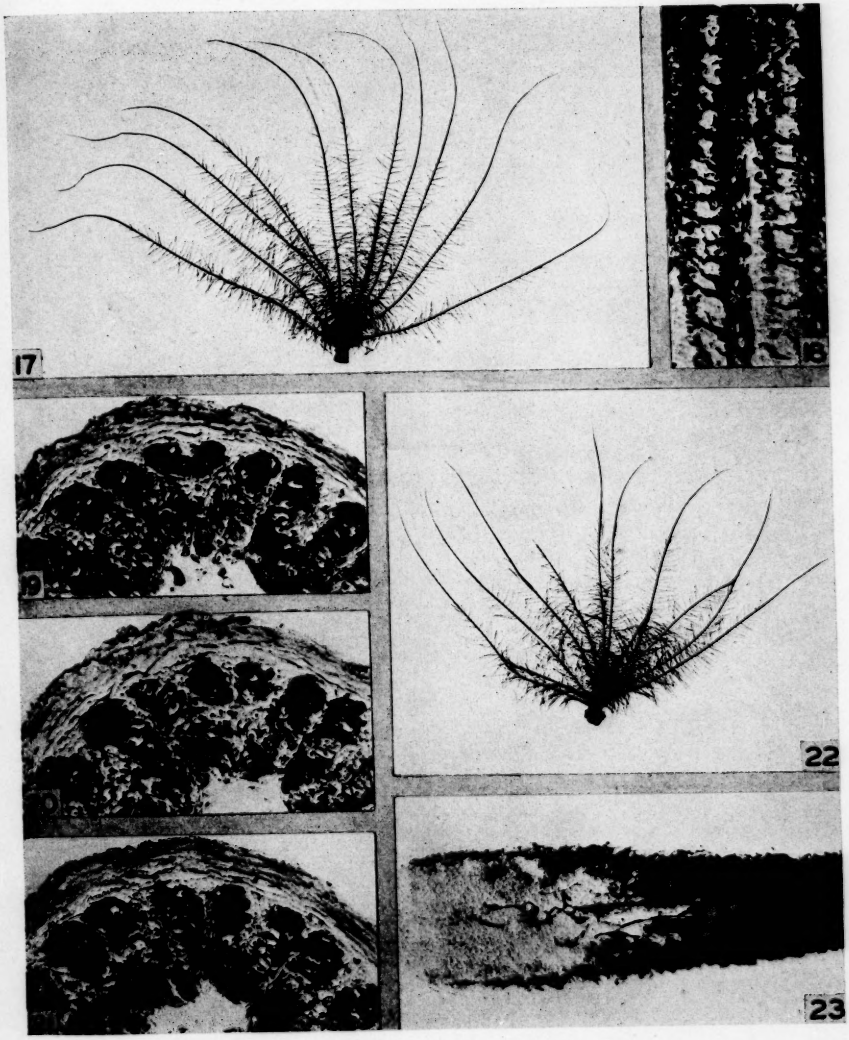
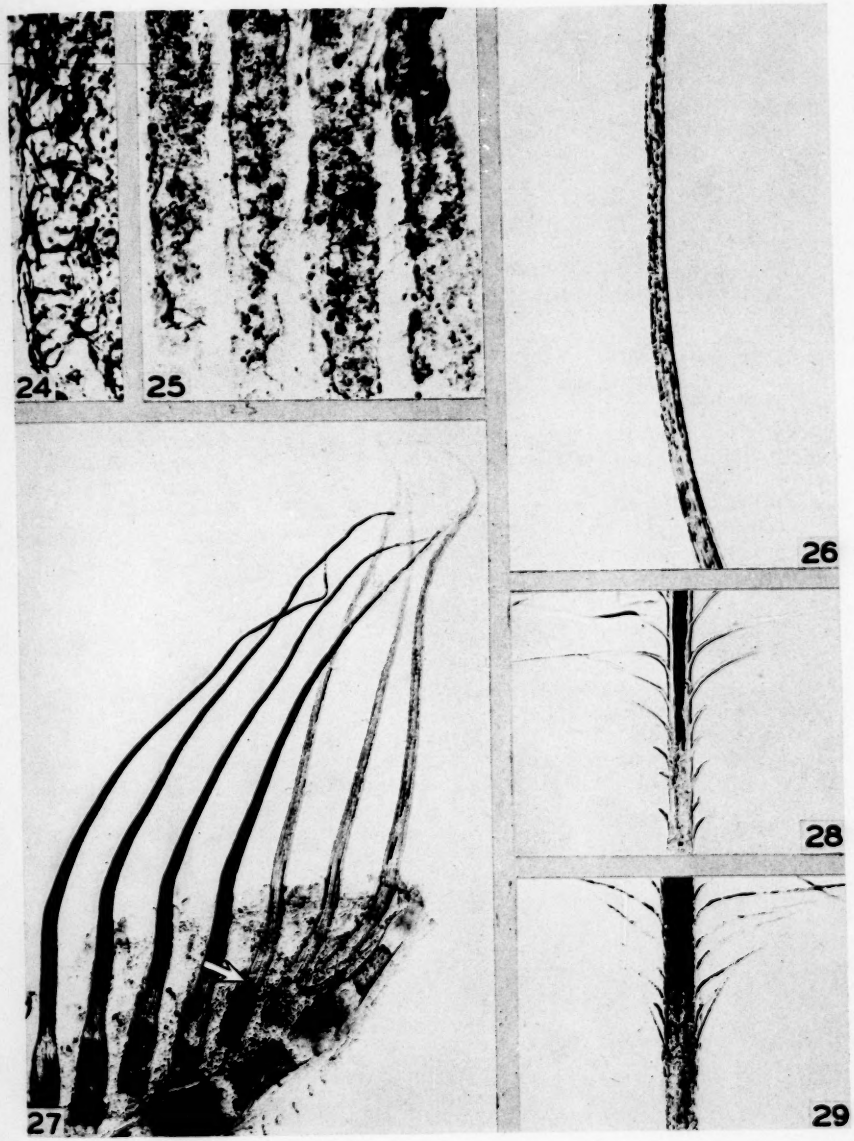


PLATE IV



## PLATE III

FIG. 17.—A down feather from the wing of a newly hatched chick. Twelve barbs extend upward from the calamus. Each barb bears two rows of barbules on its proximal two-thirds. ×5.

FIG. 18.—Two barb-vane ridges of a split feather germ from the wing of a 13-day and 5-hour embryo. Ridges viewed from pulp surface. Cell bodies of pigment cells lie along the pulp margin of each ridge. Their branched processes extend peripherally, depositing pigment into barbule cells. Note that melanophore processes lie perpendicular to the long axis of the ridges. ×210.

FIGS. 19, 20, AND 21.—Transverse sections at three different apicobasal levels of a feather germ from the wing of a 12-day female embryo. ×1,000.

FIG. 19.—A section through three barb-vane ridges. For orientation see Fig. 3. The ridge in the center undergoes splitting just proximal to this level. Note its broad base and widely divergent barbule plates. Pigment is deposited in cells of its axial plate. Contrast with adjacent ridges.

FIG. 20.—The same three ridges 28 $\mu$ proximal to Fig. 19. The single group of pigmented cells is separated into two groups by unpigmented cylinder cells. A slight indentation appears at the inner surface of the ridge.

FIG. 21.—The same region 35 $\mu$ proximal to Fig. 20. Splitting is completed. Cylinder cells adjacent to the pulp are now continuous with those lying between the two groups of pigmented cells. Cells originating in the axial plate of one barb-vane ridge are distributed between two ridges and will form barbules attached to two different barbs.

FIG. 22.—A down feather from a newly hatched chick showing three split barb-vanes which arose by the splitting process observed in Figs. 19, 20, and 21. ×4.

FIG. 23.—The base of a whole mount of an 11-day feather germ showing dermal melanophores in the pulp. These cells occur singly or as long chains. ×100.

## PLATE IV

FIG. 24.—One barb-vane ridge of a split feather germ from the wing of a $14\frac{1}{8}$-day embryo. Viewed from the pulp surface. Melanophores now lie roughly parallel to the long axis of the ridge. Contrast with Pl. III, Fig. 18. ×210.

Fig. 25.—Four barb-vane ridges of a split feather germ from a $14\frac{5}{8}$-day embryo. Viewed from the pulp surface. Degenerate melanophores are reduced to solid balls of pigment which lie at the pulp margin of each ridge. ×210.

FIG. 26.—Whole mount of the desiccated pulp and overlying cylinder cells and melanophores. Removed from the center of a down feather of a newly hatched chick. Such a strand is attached only at the calamus and is easily broken off and lost. ×40.

FIG. 27.—The tip of the wing of an embryo of $16\frac{5}{8}$-days incubation. There are three white down feathers (any black in them is due to the presence of air). Melanophores are present throughout the dermis surrounding the feather follicles. Note the abrupt appearance of pigment in the feather cylinder during the organization of the tip of the juvenile feather (*arrow*). ×10.

FIG. 28.—The base of a male barb-vane of a newly hatched chick. Note the small amount of pigment contained in the barb and barbules. ×100.

FIG. 29.—The base of a female barb-vane of a newly hatched chick. Pigment is abundant in both barb and barbules. Contrast with Fig. 28. ×100.